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A. BRZEZIŃSKI and B. FILIPOWICZ

THIAMINE AND THIAMINE ESTERS IN GUINEA PIG HEART MUSCLE IN EXPERIMENTAL DIPHTHERIAL TOXAEMIA

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Previously we have reported that in the liver of diphtheria-intoxicated guinea pig the content of cocarboxylase decreased [15] but the amount of total thiamine esters was unaltered [2]. These observations permitted to suggest that in diphtherial toxaemia the content of enzymically inactive thiamine esters, probably the monophosphate ester (TMP), is increased. Since the heart is the organ most often affected in diphtheria, the content of thiamine in the heart muscle in diphtherial toxaemia has been studied in this work.

MATERIAL AND METHODS

For experiments, male guinea pigs weighing about 250 g. were used. The diet and the method of administration of diphtherial toxin (1 DLM) were as described previously [2]. The determinations were made 48 - 60 hr. after intoxication.

Total and free thiamine were estimated fluorimetrically by the Hennessy--Cerecedo method, as described previously [2]. The amount of esterified thiamine was calculated from the values for total and free thiamine. Cocarboxylase was assayed manometrically according to Westenbrink & Steyn-Parvé [14]. For technical reasons, different animals were used for fluorimetric and manometric determinations.

For cocarboxylase determinations, 0.25 g. of the heart muscle was homogenized for 1 min. in 5 ml. of 0.1 M-phosphate buffer, pH 6.2, in a Potter-Elvehjem type homogenizer provided with a teflon pestle. The homogenate was heated for 5 min. on a boiling water bath. After cooling, it was added with buffer to a volume of 25 ml., and the filtrate was used for determinations. It has been checked that the extraction of cocarboxylase with phosphate buffer gives the same results as the extraction with dilute HCl, recommended by Westenbrink & Steyn-Parvé [14].

Alkali-washed dried brewer's seed yeast obtained from the Brewery no. 1 in Łódź, was used as apoenzyme of cocarboxylase. Dried yeast, 1 g., was suspended in 20 ml. of water by vigorous shaking for 3 min. in a stoppered vessel. To the

homogeneous suspension, 20 ml. of 0.2 M-Na_2HPO_4 adjusted to pH 10 and 0.2 ml. of 10% NaOH were added, the final pH of the mixture being about 8.8. The mixture was shaken vigorously for 5 min., centrifuged for 1 min. at 3000 rev./min. and the sediment washed three times with 40 ml. of water. Then it was added with 1 ml. of 0.1 M-$MnCl_2$, 0.25 ml. of a solution of pyrimidyl (containing 15 mg./ml.) and phosphate buffer, pH 6.2, to a final volume of 6.5 ml. Pyrimidyl (2-methyl-4-amino--5-ethoxymethylpyrimidine) was applied as an inhibitor of yeast phosphatase. The washing of yeast with alkali should be done as rapidly as possible; under the conditions used the procedure did not last longer than 25 min.

The determinations were made in a Warburg apparatus at 28°, with air as the gas phase. Berolase (Hoffman-La Roche, Switzerland) was used as cocarboxylase standard; on chromatography according to Rossi-Fanelli *et al.* [11] this preparation appeared to have a very slight contamination of TMP. The stock solution of Berolase at a concentration of 200 μg./ml. of 0.1 M-phosphate buffer, pH 5.0, was kept in a refrigerator. Just before the determinations standard solutions were prepared at concentrations of 0.05, 0.10, 0.15 and 0.20 μg./ml. of 0.1 M-phosphate buffer, pH 6.2.

The main compartment of the Warburg flask contained 1 ml. of the cocarboxylase standard or tested sample and 0.5 ml. of the apoenzyme preparation. In the side arm 0.2 ml. of 2.5% sodium pyruvate (Sigma, St. Louis, U.S.A.) in phosphate buffer

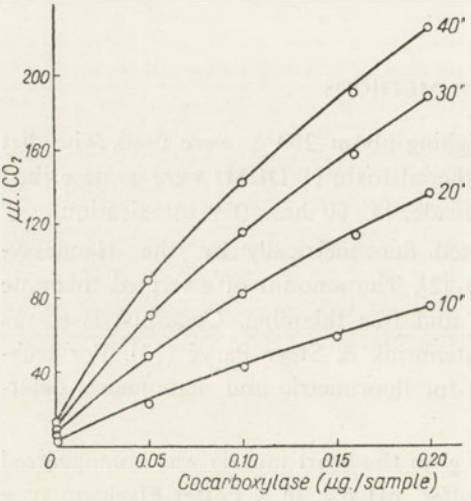

Fig. 1. The effect of cocarboxylase concentration on the enzymic decarboxylation of pyruvic acid (standard calibration curves).

were placed. After 15 min. of temperature equilibration, the pyruvate was tipped in and the readings were made after 10, 20 and 30 min. The concentration of the sample tested was always between the concentrations of the standard solutions. All determinations were done on at least two parallel samples. The amount of cocarboxylase in the tested samples was calculated from the standard calibration curve (Fig. 1) taking the average from values for 10, 20 and 30 min. of incubation. The amount of cocarboxylase was then calculated per gram of wet weight of the heart muscle.

RESULTS

The content of total thiamine determined fluorimetrically (Table 1) in the heart muscle of the normal guinea pig, amounted to 6.6 μg./g.; only a very small part of it, less than 5%, appeared in the free form; the rest, about 95% (6.3 μg./g.) was esterified and was released after treatment with Taka-Diastase. In intoxicated animals, the content of total thiamine increased to about 7.9 μg./g. As the amount of free thiamine was unaltered, it follows that the amount of esterified thiamine increased. This increase, according to Student's *t* test, was statistically significant

Table 1

Content of free and esterified thiamine in the heart muscle of normal and diphtheria-intoxicated guinea pigs

Thiamine was determined fluorimetrically. The figures represent μg. of thiamine per g. of fresh tissue. Mean values ±S.D. are given and in parentheses the number of experiments.

Compound	Control animals	Intoxicated animals
	(μg./g. fresh tissue)	
Total thiamine	(6) 6.6±0.26	(7) 7.9±0.37
Free thiamine	(6) 0.3±0.09	(7) 0.24±0.05
Esterified thiamine	(6) 6.3±0.3	(7) 7.7±0.38

Similar results were obtained by manometric determinations (Table 2) of the coenzymically active thiamine esters, first of all TDP. In the heart muscle of the normal guinea pig, the content of cocarboxylase amounted to about 8.5 μg./g. which, calculated as molar concentration (Table 3), corresponded to the amount of esterified thiamine. It would follow that in the heart muscle of normal animals all or almost all bound thiamine was in the coenzyme form. The values obtained are somewhat lower than those of Westenbrink & Steyn-Parvé [13] who reported the values of 9.0 and 9.7 μg./g., found in two animals. In intoxicated animals the content of cocarboxylase was higher by about 25%, reaching on the average 10.7 μg./g. Among the intoxicated animals, four were tested while in agonal state

Table 2

Content of cocarboxylase in the heart muscle of normal and diphtheria-intoxicated guinea pigs

Cocarboxylase was determined manometrically. Mean values ± S.D. and in parentheses the limit values are given.

Animals	No. of determinations	Cocarboxylase (μg./g. fresh tissue)
Control	8	8.54±0.72 (7.3 - 9.7)
Intoxicated	10	10.71±0.46 (10.0 - 11.4)
In agonal state	4	9.2 (8.0; 9.5; 9.6; 9.7)
After death	2	8.0 (7.9; 8.1)

Table 3

Content of free and esterified thiamine and of cocarboxylase in the heart muscle of normal and diphtheria-intoxicated guinea pigs

The values are calculated from the data presented in Tables 1 and 2. Mean values ±S.D. are given and in parentheses the number of determinations.

Compound	Control animals (mμmoles/g. fresh tissue)	Intoxicated animals (mμmoles/g. fresh tissue)	Student's *P* value
Total thiamine	(6) 21.4±0.8	(7) 25.5±1.2	< 0.001
Free thiamine	(6) 1.0±0.3	(7) 0.8±0.16	= 0.7
Esterified thiamine	(6) 20.4±1.0	(7) 24.9±1.2	< 0.001
Cocarboxylase	(8) 20.4±1.72	(10) 25.8±1.1	< 0.001

and two just after death. In these animals, the content of cocarboxylase was lower, being similar or equal to that in the normal guinea pig. Similar results were obtained by the fluorimetric method; the content of esterified thiamine in two animals tested in agonal state was even lower than in normal animals.

DISCUSSION

It is well known that diphtheria very often affects the cardiovascular system; for instance Kuźniecow [6] among 241 adult patients, in 64% observed impaired circulation. For children, Ladbetter & Cannon [7] and Skwarczewska-Stypułkowska [12] reported somewhat lower figures. The frequency of cardiovascular damage depends mainly on the course of the disease [5] ranging from 5% in the mild forms to 100% in hypertoxic diphtheria. Moreover, in diphtheria the cardiovascular failure is most frequently the cause of death.

From the data presented in this work it appears that in diphtheria-intoxicated guinea pigs within 48 - 60 hr. after intoxication, the content of total and esterified thiamine in the heart muscle increased by 22%, and the content of cocarboxylase by 25%. This is probably due to the increased activity of the heart muscle accompanied by an increase in energy requirement and accelerated metabolism. It may also depend to some extent on metabolic adjustments. Miszukowa & Lebedewa [10] reported that in the heart muscle of diphtheria-intoxicated rabbits a half of the pyruvic acid is oxidized to CO_2 and H_2O, whereas in normal animals only one-fifth. It seems possible that for the diphtheria-dependent increase of pyruvic acid oxidation the amount of available cocarboxylase is insufficient and probably thiamine from other tissues accumulates in the heart muscle. It seems also possible that the therapeutic effect of cocarboxylase consists in diminishing this deficiency. The lack of any therapeutic effect of free thiamine administration could be explained by a deficiency of ATP for the synthesis of cocarboxylase, as suggested among others by Cessi *et al.* [3] and Barbieri *et al.* [1]. When the organism is no longer able to maintain the concentration of cocarboxylase at an adequate level, there appears the condition called by Hegglin [4] "the energetic failure of the cardiac muscle"

with all its fatal consequences. This view seems to be supported by the determinations made on animals in agonal state or just after death. Such mechanism would be also in agreement with the observations of Lasch [8, 9] who by administering cocarboxylase achieved a marked decrease of deaths from diphtherial toxaemia in children.

SUMMARY

In the heart muscle of the normal guinea pig the content of total thiamine amounted to about 6.6 μg./g. of fresh tissue, about 95% of thiamine being in cocarboxylase form. In experimental diphtherial toxaemia, the amount of total thiamine was higher by 25% due to an increase in the content of cocarboxylase. This could be connected with the increased activity of the heart muscle. The decrease in the content of cocarboxylase observed in agonal state may be considered as one of the factors leading to the energetic failure of the heart muscle.

REFERENCES

[1] Barbieri A., Spialtini A. & Riccardi B. - *Atti di VI Congresso Int. di Microbiologia* **1**, 745, 1953.
[2] Brzeziński A. & Filipowicz B. - *Acta Biochim. Polon.* **11**, 323, 1964.
[3] Cessi C., Montanaro L. & Serafini-Cessi F. - *Biochem. J.* **87**, 35P, 1963.
[4] Hegglin R. - *Schweitz. Med. Wsch.* **46**, 1103, 1953.
[5] Iwanowa S. S. - *Pediat. Akush. Ginek.* (Ukr.) **5**, 23, 1962.
[6] Kuźniecow W. S. - *Terap. Archiv.* **31**, 77, 1959.
[7] Ladbetter M. K. & Cannon A. B. - *Amer. J. Diseases Child.* **100**, 608, 1960.
[8] Lasch F. - *Annales Paed.* **5/6**, 333, 1952.
[9] Lasch F. - *Dt. Med. Wsch.* **27/28**, 975, 1953.
[10] Miszukowa E. A. & Lebedewa L. N. - *Woprosy Med. Chim.* **6**, 275, 1960.
[11] Rossi-Fanelli A., Siliprandi N. & Fasella P. - *Science* **116**, 771, 1952.
[12] Skwarczewska-Stypułkowska H. - *Pediatria Pol.* **34**, 1217, 1959.
[13] Westenbrink H. G. K. & Steyn-Parvé E. - *Biochim. Biophys. Acta* **1**, 87, 1947.
[14] Westenbrink H. G. K. & Steyn-Parvé E. - *Int. Z. Vitaminforsch.* **21**, 461, 1950.
[15] Witkowski S., Brzeziński A., Gałamon T. & Filipowicz B., *Intern. Symposium on B Vitamins*, p. 273, Poznań 1959.

TIAMINA I JEJ ESTRY W MIĘŚNIU SERCOWYM ŚWINEK MORSKICH W DOŚWIADCZALNEJ TOKSEMII BŁONICZEJ

Streszczenie

W mięśniu sercowym zdrowych świnek morskich poziom tiaminy całkowitej wynosi około 6,6 μg/g. świeżej tkanki, z tego około 95% w postaci kokarboksylazy. W eksperymentalnej toksemii błoniczej ilość tiaminy całkowitej była wyższa o około 25% w związku ze wzrostem ilości kokarboksylazy. Przypuszczalnie jest to związane ze zwiększoną pracą mięśnia sercowego. Zmniejszenie zawartości kokarboksylazy obserwowane w okresie przedzgonnym może być uważane za jeden z czynników doprowadzających do tzw. energetycznej niedomogi mięśnia sercowego.

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ACTIVITY OF TRANSKETOLASE IN GUINEA PIG TISSUES IN EXPERIMENTAL DIPHTHERIAL TOXAEMIA

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Changes in the content of total and esterified thiamine and of cocarboxylase in the liver and heart of diphtheria-intoxicated guinea pigs, have been reported previously [12, 3, 4]. In the present work an attempt was made to determine whether these changes affect the enzymic reactions dependent on thiamine diphosphate. As it is known [1] that transketolase activity is the most sensitive index of vitamin B_1 deficiency, this activity was determined in tissues of diphtheria-intoxicated guinea pigs.

METHODS

For experiments, male guinea pigs, weighing about 250 g., were used. The diet and the method of administration of diphtherial toxin (1 DLM) were the same as described previously [3]. The determinations were made 48 - 60 hr. after intoxication.

The transketolase activity was determined according to Sie *et al.* [9]. These authors observed [8, 10] the conversion of nucleoside to sedoheptulose in tissue extracts. In this system the nucleoside probably undergoes phosphorolysis and the phosphopentoses formed, after isomerization and epimerization, serve as substrate for transketolase.

The animals were killed by decapitation and the tissues to be tested were removed rapidly, rinsed with cold 1.22% KCl and homogenized for 45 sec. in a Potter-Elvehjem homogenizer with a teflon pestle, in 0.01 M-phosphate buffer (Na_2HPO_4/KH_2PO_4), pH 6.7, to form 10% homogenates. The homogenate was centrifuged for 30 min. at 18 000 *g* in a Servall centrifuge at 0 - 4°. The clear supernatant fluid, called the enzyme, was used for assays.

The incubation mixture consisted of the enzyme solution (0.8 ml. of heart or brain extract, 0.3 ml. of extract from other tissues); 0.5 ml. of 0.035 M-adenosine (Hoffman-La Roche, Switzerland) in 0.01 M-phosphate buffer, pH 6.7, and in the experiments with the kidney, liver, spleen and lung, additionally 0.5 ml. of the buffer.

The mixture was incubated for 4 hr. at 37° under toluene, then added with 7.5 ml. of 96% ethanol and kept for 30 min. in a refrigerator. Next the mixture was transferred to a centrifuge tube and the incubation tube rinsed twice with 3.1 ml. of water. To the pooled fluids (15 ml.), 3 ml. of concentrated 12 N-HCl was added, mixed, centrifuged for 15 min. at about 10 000 *g* and the sedoheptulose formed was determined in the supernatant. In the control sample, enzyme solution was added after ethanol and the sample was immediately placed in the refrigerator.

Sedoheptulose was determined according to Dische [6] with a modification consisting in using copper ion instead of ferrum ion [5]. This permitted to obtain a deeper colour and more advantageous relationship between ion concentration and extinction. It was observed that the presence of ethanol, used by Sie *et al.* [9] to denature protein, during 1-hr. heating of the sample with hydrochloric acid may cause even a twofold increase in extinction. It should be noted that if the ethanol was added to the standard sample after acid hydrolysis, just before the heating with orcinol, the extinction was lower whereas the increase of alcohol concentration

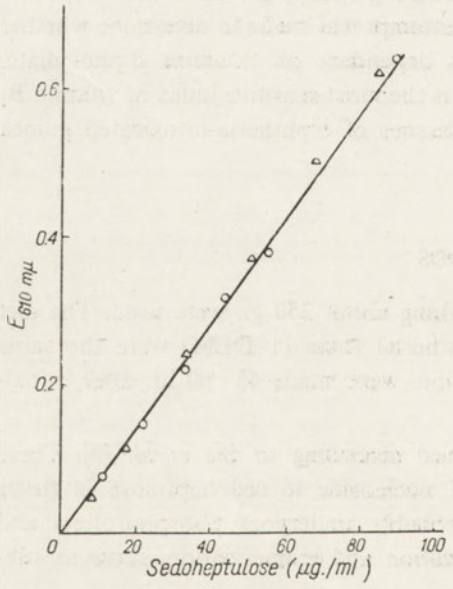

Fig. 1. The relationship between sedoheptulose concentration and the orcinol colour reaction, in a modification of the Dische method (for details see text). Sedoheptulose (Sigma, St. Louis, U.S.A.) was used as standard. (○), Without adenosine; (Δ), after addition of adenosine.

resulted in a shift of the extinction maximum from 570 to 630 mμ. In the standard solution a linear relationship was obtained between the extinction and sedoheptulose concentration over a range of at least 10 - 100 μg. (Fig. 1). It has been determined that in the procedure applied even a tenfold excess of adenosine does not affect the extinction.

The following procedure was used: 4 ml. of acid supernatant was heated for 1 hr. on a boiling water bath in calibrated tubes closed with glass stoppers, the upper parts of the tubes being cooled with a stream of air. After heating, the samples were cooled, adjusted if necessary with ethanol to the volume of 4 ml. and then added with 0.4 ml. of 6% orcinol in 96% ethanol and 0.4 ml. of 0.05 M-$CuSO_4$

in 2 N-HCl. After mixing, the tubes were closed, placed in a boiling water bath for exactly 3 min., cooled, and the extinction was determined in a Unicam SP 500 spectrophotometer in 1 cm. light-path glass cuvettes at 610 mμ. The amount of sedoheptulose was calculated from the extinction after subtracting the value for the control sample, then the activity of transketolase was expressed as μmoles of sedoheptulose formed per 1 g. of fresh tissue.

RESULTS AND DISCUSSION

Transketolase activity was assayed in the heart muscle, brain, lung, liver, kidney and spleen of normal and diphtheria-intoxicated guinea pigs (Table 1). It should be noted that there was no correlation between the transketolase activity and the content of esterified thiamine or cocarboxylase in the examined tissues. In normal animals, the content of cocarboxylase determined according to Westenbrink & Steyn-Parvé [11] has been found to be lower in the liver (4.88 μg./g. [12]) than in the heart muscle (8.54 μg./g. [4]), whereas the transketolase activity in the liver was several times higher than in the heart muscle.

Table 1

Transketolase activity in the tissues of normal and diphtheria-intoxicated guinea pigs

The figures represent μmoles of sedoheptulose formed per 1 g. of fresh tissue. Mean values ±S.D. and in parentheses the limit values are given.

Tissue	Control animals		Intoxicated animals		Student's *P* value
	No. of expt.	(μmoles/g.)	No. of expt.	(μmoles/g.)	
Heart	9	19.2±1.31 (16.9 - 20.4)	9	27.0±1.85 (24.3 - 29.5)	< 0.001
Brain	9	16.8±1.77 (15.2 - 20.9)	8	13.3±1.74 (10.0 - 16.0)	< 0.001
Liver	8	122.5±33.8 (78.1 - 166.5)	9	74.5±18.1 (53.1 - 113.5)	< 0.01
Spleen	9	310.5±45.5 (238.0 - 381.0)	9	182.2±30.8 (112.5 - 224.5)	< 0.001
Kidney	9	99.9±18.5 (70.8 - 137.3)	9	113.2±18.5 (89.5 - 151.0)	$0.2 > P > 0.1$
Lung	9	156.5±20.1 (129.8 - 194.5)	9	145.9±16.0 (113.0 - 165.5)	> 0.2

After intoxication, the transketolase activity in the kidney and lung was unaltered. In the brain, liver and spleen the activity was lower, and in the heart muscle it was higher than in normal animals. The differences were statistically significant.

Changes in transketolase activity in the liver and in heart muscle were parallel to the previously observed changes in diphosphothiamine content. In the liver of intoxicated animals the transketolase activity was lower by about 40%, the corresponding decrease of cocarboxylase being about 27% (from 4.88 to 3.54 μg./g. fresh tissue [12]). In the heart muscle the transketolase activity was higher by about 40% and the increase in the content of cocarboxylase was 25% [4]. It was previously suggested [4] that the increased content of cocarboxylase in the heart muscle of

intoxicated animals (and the accompanying increase in transketolase activity) may be due to accumulation of the available thiamine in the heart muscle whereas the lower content of cocarboxylase in the liver after intoxication could be caused by increased activity of thiamine pyrophosphatase decomposing a part of thiamine diphosphate to thiaminemonophosphate [3]. The decrease in transketolase activity observed also in other tissues (brain, spleen) may have a mechanism similar to that suggested for the liver or it may be connected with the accumulation of thiamine in the heart muscle.

The decrease in transketolase activity found in the brain is at variance with the observations on avitaminosis B_1. Mochnacka & Szafrański [7] found no decrease in transketolase activity in the brain of vitamin B_1 deficient pigeons. Brin [2] studied transketolase activity in rats kept on a thiamine-deficient diet and found that already after four days the content of thiamine decreased in the heart muscle and after about a week, in other tissues. Brin, during 13 days of the experiment, observed no decrease of activity in the brain, even after additional administration of oxythiamine. These authors concluded that the brain tissue is comparatively resistant to thiamine deficiency and that even a marked decrease of the content of thiamine in other tissues does not affect the transketolase activity in the brain. Sie [8] arrived at similar conclusions.

Quite a different picture was observed in diphtherial intoxication: the transketolase activity increased markedly in the heart muscle whereas in the brain tissue it decreased by about 20%.

The differences between the transketolase activity in vitamin B_1 deficiency and in diphtherial toxaemia, together with the previously observed changes in the content of esterified thiamine and thiamine diphosphate in the liver and heart muscle, may indicate that in diphtherial intoxication these changes are not related to thiamine deficiency. The changes observed may be due to an increase of thiaminepyrophosphatase activity, mainly in the liver, and to accumulation of thiamine in the heart muscle. The results obtained seem to confirm the advisability of cocarboxylase administration in the treatment of diphtheria, and especially of diphtherial toxaemia.

SUMMARY

In the normal guinea pig the highest transketolase activity was found in the spleen > lung > liver > kidney > heart and brain. In intoxicated animals a statistically significant increase of activity was found in the heart muscle (40%) and a decrease by 40% in the liver and spleen, and by 20% in the brain. No changes were found in the kidney and lung.

REFERENCES

[1] Brin M. - *Ann. N.Y. Acad. Sci.* **98**, 528, 1962.
[2] Brin M. - *J. Nutr.* **78**, 179, 1962.
[3] Brzeziński A. & Filipowicz B. - *Acta Biochim. Polon.* **11**, 323, 1964.

[4] Brzeziński A. & Filipowicz B. - *Acta Biochim. Polon.* **12**, 279, 1965.
[5] Brzeziński A. & Więckowski W., - *I Kraj. Kongres Biochemii*, p. 178, Łódź 1964.
[6] Dische Z. - *J. Biol. Chem.* **204**, 983, 1953.
[7] Mochnacka I. & Szafrański P. - *Acta Biochim Polon.* **3**, 539, 1956.
[8] Sie H. G. - *Proc. Soc. Exp. Biol. Med.* **113**, 733, 1963.
[9] Sie H. G., Nigam V. N. & Fishman W. H. - *J. Biol. Chem.* **234**, 1202, 1959.
[10] Sie H. G., Nigam V. N. & Fishman W. H. - *Biochim. Biophys. Acta* **50**, 277, 1961.
[11] Westenbrink H. G. K. & Steyn-Parvé E. - *Int. Z. Vitaminforsch.* **21**, 461, 1950.
[12] Witkowski S., Brzeziński A., Gałamon T. & Filipowicz B. - *Intern. Symposium on B Vitamins*, p. 273, Poznań 1959.

AKTYWNOŚĆ TRANSKETOLAZY W PRZEBIEGU DOŚWIADCZALNEJ TOKSEMII BŁONICZEJ U ŚWINEK MORSKICH

Streszczenie

U zdrowych świnek morskich najwyższą aktywność transketolazową ma śledziona, a następnie kolejno płuca, wątroba, nerki, mięsień sercowy i mózg. U świnek intoksykowanych jadem błoniczym obserwuje się statystycznie znamienny wzrost aktywności transketolazowej mięśnia sercowego (o ok. 40%) oraz spadek aktywności w wątrobie (o ok. 40%), mózgu (o ok. 22%) i śledzionie (o ok. 40%). W nerkach i płucach nie spostrzeżono zmian aktywności.

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THE INFLUENCE OF ENERGY ON THE REDUCTION OF UBIQUINONE IN OX HEART MITOCHONDRIA

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Various flavoprotein enzymes such as succinate-, NADH- [3], glycero-1-phosphate- [9] and choline- [8] dehydrogenases are able to reduce mitochondrial ubiquinone. It has been generally accepted that ubiquinone is located in the respiratory chain between flavoproteins and the cytochrome system [4]. However, its oxido-reduction changes indicate that this scheme might not be so simple. Direct measurements in a double-beam spectrophotometer showed that the degree of reduction of ubiquinone depends upon the tightness of respiratory coupling of the prepared mitochondria [10].

The purpose of this work was to investigate the effect of ATP and of uncoupling agents on the oxido-reduction state of ubiquinone in ox heart mitochondria.

METHODS AND MATERIALS

Ox heart mitochondria were prepared in a sucrose-phosphate-EDTA medium as described by Crane *et al.* [1], washed once with 0.25 M-sucrose - 0.001 M-EDTA and finally suspended in the same solution. All the experiments were carried out in 0.25 M-sucrose - 0.02 M-TRA (triethanolamine hydrochloride) - 0.001 M-EDTA medium, pH 7.2, at 20°. Detailed conditions are given in the legends for the Tables. The respiratory control was determined with Clark oxygen electrode in the same medium. Oxidized and reduced ubiquinone was determined essentially according to the procedure of Pumphrey & Redfearn [7] except that methanol alone was used for deproteinization at 0°. (Assays carried out on ox heart and rat liver mitochondria showed that there was no difference in results obtained after deproteinization either with methanol alone or with methanol and pyrogallol). The incubation mixture was extracted three times with light petroleum (b.p. 40 - 50°), the extracts collected, washed once with 95% methanol and centrifuged. The light petroleum layer was transferred to a small beaker and evaporated to dryness at 37° under a stream of nitrogen. The residue was dissolved in 1.2 ml. of spectroscopically pure ethanol, and oxidized ubiquinone determined spectrophotometrically at 275 mμ.

The amount was calculated from the decrease in extinction after the addition of a few crystals of potassium borohydride. Millimolar extinction coefficient $\Delta\varepsilon_{275\,m\mu}$ 12.25 cm.$^{-1}$ [5] was used. Protein was determined by the biuret method as described by Szarkowska & Klingenberg [10] using the formula: mg. protein $= \Delta E_{546\,m\mu} \times$ $\times 17.5$ for the volume of 5 ml. and the light path of 1 cm.

All the chemicals used were commercial products. ATP (Pabst Lab. Milwaukee, Wisc., USA), antimycin A (Sigma, St. Louis, Mo., USA).

RESULTS

In freshly prepared ox heart mitochondria, aerated to prevent anaerobiosis, ubiquinone was present mainly in the oxidized form. The addition of succinate to the incubation mixture caused an appreciable reduction of ubiquinone up to 50 - 60% (Table 1). The effect of the other substrates was much smaller and both malate and α-ketoglutarate induced but a slight reduction of the mitochondrial ubiquinone (about 10%). If ATP was added to the incubation mixture, with succinate as substrate, the reduction of ubiquinone increased and more of the reduced form was found. This effect of ATP was not noticeable if, instead of succinate, NAD-dependent substrates were used.

Table 1

The influence of ATP on the reduction of ubiquinone

Ox heart mitochondria in 0.25 M-sucrose - 0.02 M-TRA - 0.001 M-EDTA medium, pH 7.2. After preliminary 5 min. incubation with aeration the substrate was added (5 mM) and the incubation continued for the next 5 min. in aerobic conditions. If α-ketoglutarate was used as substrate, malonate was added in the same concentration. ATP in 2 mM concentration was added together with substrate.

Additions	Oxidized ubiquinone (μmoles/g. protein)	
	Expt. 1	Expt. 2
None	4.28	5.41
ATP	4.28	5.41
Succinate	1.68	2.66
Succinate and ATP	1.01	1.77
α-Ketoglutarate	3.70	4.85
α-Ketoglutarate and ATP	—	4.80
Malate	3.26	4.97
Malate and ATP	3.26	4.84

The data presented in Table 2 show that two well-known uncoupling agents of the oxidative phosphorylation, i.e. $CaCl_2$ and 2,4-dinitrophenol (DNP), induced oxidation of ubiquinone previously reduced by succinate. In the presence of both the uncoupler and the succinate the degree of reduction of ubiquinone was equal to that reached in the absence of the substrate.

Table 2

The effect of uncouplers on the reduction of ubiquinone

Medium and substrate concentration as in Table 1. $CaCl_2$ 2 mM (above the concentration of EDTA), DNP 0.1 mM. The experiment was carried out in aerobic conditions. After 5 min. preincubation the substrate was added, after another 2 min. the uncoupling agent, and the incubation was continued for the next 5 min.

Additions	Oxidized ubiquinone (μmoles/g. protein)	
	Expt. 1	Expt. 2
None	4.86	5.41
$CaCl_2$	4.73	5.20
DNP	5.21	5.36
Succinate	2.79	2.90
Succinate; $CaCl_2$	5.24	5.25
Succinate; DNP	5.14	5.00

The addition of antimycin A (AA) at a concentration which blocks the electron transport chain, to mitochondria uncoupled by the presence of $CaCl_2$ or dinitrophenol, resulted in reduction of ubiquinone (Table 3). Although this reduction was very rapid, it was not complete; an additional increase in the state of reduction of ubiquinone was observed upon a subsequent addition of KCN or ATP. Since ATP added after KCN did not cause any further changes in the reduction of ubiquinone, one might suppose that in the presence of either KCN or ATP the same pool of ubiquinone became reduced. These observations are valid both for succinate and malate as substrate.

In the experiment presented in Table 4, ubiquinone had been initially reduced by succinate under anaerobic conditions, then malonate was added to cut off the source of hydrogen, and the incubation mixture was transferred to aerobic conditions. It can be seen (Expt. no. 1) that in tightly-coupled mitochondria antimycin A inhibited completely oxidation of the previously reduced ubiquinone. However, in the presence of uncoupler, even when the electron transport chain was blocked with antimycin A, ubiquinone became partially oxidized. In experiment no. 2 the incubation mixture was preincubated for 5 min. in aerobic conditions to exhaust the endogenous substrates and high-energy intermediates. It can be seen that in the absence of dinitrophenol antimycin A inhibited only partially oxidation of the previously reduced ubiquinone, while in the presence of dinitrophenol all the ubiquinone became oxidized. When ATP was added to the incubation mixture it prevented oxidation of ubiquinone (Table 4, expt. 3).

DISCUSSION

The presented results indicate that the degree of reduction of ubiquinone depends at least to some extent upon the addition of energy. The addition of ATP causes a shift of equilibrium towards the reduction of ubiquinone, and the addition of

uncoupling agents $CaCl_2$ and dinitrophenol, towards its oxidation. Szarkowska & Klingenberg [10] directly observed conspicuous changes in absorption due to the oxidation of ubiquinone after the addition of $CaCl_2$ to rat heart mitochondria in a controlled state.

Table 3

The reduction of ubiquinone in the presence of antimycin A and the uncouplers

After 5 min. of preincubation the substrate (5 mM) was added and the incubation carried out for 2 min. in the case of succinate and for 5 min. in the case of malate. Then the uncoupling agent ($CaCl_2$, 2 mM or DNP, 0.1 mM) was introduced and the incubation continued for 5 min. Then antimycin A (AA) was added (0.8 μg. per mg. protein) where indicated and after 3 min. KCN in a final concentration of 1 mM, followed by ATP (2 mM).

Additions	Oxidized ubiquinone (μmoles/g. protein)		
	Expt. 1	Expt. 2	
None	4.75	4.55	
$CaCl_2$	5.03	4.47	
Succinate	2.79	2.90	
Succinate; $CaCl_2$	4.96	4.63	
Succinate; $CaCl_2$; AA	2.70	1.83	
Succinate; $CaCl_2$; AA; KCN	1.19	1.07	
Malate	4.03	—	
Malate; $CaCl_2$	4.96	—	
Malate; $CaCl_2$; AA	4.31	—	
Malate; $CaCl_2$; AA; KCN	2.90	—	
	Expt. 1	Expt. 2	Expt. 3
None	5.73	4.29	4.54
DNP	6.07	4.27	5.81
Succinate	3.23	1.86	—
Succinate; DNP	6.07	4.22	—
Succinate; DNP; AA	5.02	1.86	—
Succinate; DNP; AA; KCN	2.66	1.12	—
Succinate; DNP; AA; ATP	3.50	0.97	—
Succinate; DNP; AA; KCN; ATP	2.74	1.26	—
Malate	—	—	4.73
Malate; DNP	—	—	5.81
Malate; DNP; AA	—	—	3.70
Malate; DNP; AA; KCN	—	—	3.01
Malate; DNP; AA; ATP	—	—	2.70
Malate; DNP; AA; KCN; ATP	—	—	2.98

The oxido-reduction behaviour of ubiquinone under the influence of ATP and the uncoupling agents is not in discrepancy with its postulated role in the respiratory chain. The increased reduction of ubiquinone after the addition of ATP with succinate as substrate can be interpreted as the result of reversal of the electron transport

through the respiratory chain, as it was observed by Penefsky [6] when ascorbate and tetramethyl-*p*-phenylene diamine were used as substrates. However, the incomplete reduction of ubiquinone in uncoupled mitochondria in the presence of antimycin A and its further reduction after the addition of KCN or ATP, complicates this picture. ATP gave a much higher increase in reduction of ubiquinone in these mitochondria in which in the presence of antimycin A alone only a slight reduction took place, than in those in which the initial reduction in the presence of antimycin A had been already high (Table 3). It may be therefore supposed that the degree of reduction of ubiquinone in the presence of antimycin A depends upon the presence of the high-energy intermediates of the oxidative phosphorylation which even in the presence of uncoupling agents have not been completely exhausted.

Table 4

The oxidation of ubiquinone in the presence of antimycin A

Medium and substrate concentration as in Table 1. Succinate and antimycin A where indicated were present in the incubation mixture from the beginning. The incubation was carried out under nitrogen for 5 min.; at the end of this period malonate was added in a final concentration of 10 mM. Then two of the samples were transferred to aerobic conditions and the incubation was continued for the next 10 min. The experiment was repeated under the same conditions but in the presence of 0.1 mM-DNP. In expt. 2 the incubation mixture was aerated for 5 min. prior to the addition of succinate and the procedure followed as described above. Expt. 3 was performed essentially as expt. 2 except that 0.1 mM-DNP was added to all the samples and 2 mM-ATP where indicated.

Substrate and addition	Incubation 5 min. under N_2, then addition of (10 mM)	Incubation 10 min. under	Oxidized ubiquinone (μmoles/g. protein)					
			Expt. 1		Expt. 2		Expt. 3	
			No DNP	With DNP	No DNP	With DNP	With DNP	With DNP+ATP
Succinate	malonate	nitrogen	2.35	1.75	2.11	2.11	2.18	2.02
Succinate, AA	malonate	nitrogen	1.90	1.70	2.18	2.18	2.20	2.20
Succinate	malonate	air	4.17	2.75	5.11	5.30	4.80	3.28
Succinate, AA	malonate	air	2.00	3.26	3.74	5.05	4.46	2.60

The presented results could be easier explained by accepting the existence of a branched pathway of the respiratory chain on the oxygen side of ubiquinone. One of these parallel pathways appears to be antimycin A-insensitive. In such case the addition of KCN after antimycin A would cause further reduction of ubiquinone in consequence of blocking of the antimycin A-insensitive pathway. The shift of equilibrium towards the reduction of ubiquinone under the influence of ATP would take place by the reversal of the electron transport. This view is supported by the data of Table 4 from which it appears that oxidation of the previously reduced ubiquinone depends on the respiratory control of the antimycin A-insensitive branch. The existence of such a branch of the respiratory chain

is suggested by the sigmoid shape of the inhibition curves of succinate oxidase activity in relation to antimycin A concentration, obtained by Estabrook [2] in rat liver mitochondria. These studies preclude also the possibility that dinitrophenol releases antimycin A inhibition.

In mitochondria in which their original structure has been damaged by freezing, the reduction of ubiquinone does not depend upon the supply of energy. The same reduction in the presence of antimycin A as in the presence of KCN (L. Szarkowska & M. Erecińska, unpublished data) can be found, in agreement with observations of other authors.

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SUMMARY

The addition of succinate to ox heart mitochondria induces a pronounced reduction of the mitochondrial ubiquinone. ATP increases the degree of reduction of ubiquinone while the uncoupling agents induce its oxidation. In mitochondria partially uncoupled and devoid of high-energy intermediates either by a preincubation or by the addition of uncouplers, antimycin A does not ensure the complete reduction of ubiquinone. An additional reduction can be obtained after the addition of KCN or ATP.

REFERENCES

[1] Crane F. L., Glenn J. L. & Green D. E. - *Biochim. Biophys. Acta* **22**, 475, 1956.

[2] Estabrook R. W. - *Biochim. Biophys. Acta* **60**, 236, 1962.

[3] Green D. E., in *Ciba Foundation Symposium on Quinones in Electron Transport Chain* (G. E. W. Wolstenholme & C. M. O'Connor, eds.) p. 130, J. & A. Churchill Ltd., London 1961.

[4] Green D. E., *Vth Intern. Congress of Biochemistry, Moscow 1961*, Proc. vol. IX, p. 9. Structure and Function of Subcellular Particles. Pergamon Press, Oxford-London-New York-Paris 1963.

[5] Hemming F. W., *Ph. D. Thesis.* Univ. of Liverpool (1958); cit. after [7].

[6] Penefsky H. S. - *Biochim. Biophys. Acta* **58**, 619, 1962.

[7] Pumphrey A. M. & Redfearn E. R. - *Biochem. J.* **76**, 61, 1960.

[8] Szarkowska L. - *Postępy Biochemii* **10**, 77, 1964.

[9] Szarkowska L. & Drabikowska A. K. - *Life Sciences* No. 7, 519, 1963.

[10] Szarkowska L. & Klingenberg M. - *Biochem. Z.* **338**, 674, 1963.

WPŁYW ENERGII NA REDUKCJĘ UBICHINONU W MITOCHONDRIACH SERCA WOŁU

Streszczenie

Dodanie bursztynianu do mitochondrii serca wołu powoduje znaczną redukcję mitochondrialnego ubichinonu. ATP powoduje wzrost stopnia redukcji ubichinonu, podczas gdy czynniki rozkojarzające — jego utlenienie. W mitochondriach częściowo rozkojarzonych i pozbawionych wysokoenergetycznych intermediatów przez dłuższą preinkubację albo przez dodanie czynników rozkojarzających, antymycyna A nie daje całkowitej redukcji ubichinonu, którą można osiągnąć przez dodanie ATP bądź KCN.

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J. BUCZEK

RESPIRATION AND WATER ABSORPTION IN POTATO TUBERS DURING AND AFTER THE RESTING PERIOD

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Auxin-induced absorption of water by potato tuber slices is connected with respiration and can be suppressed by inhibitors of respiration [2, 7, 8, 16]. Oxygen uptake by freshly cut slices from potatoes which emerged from dormancy, kept in water in aerobic conditions, proceeds at first, during the lag phase, mainly through cytochrome oxidases [5, 17, 6, 13] reaching a maximum at the end of the lag phase (after about 48 hr.).

On the other hand, in resting potatoes the respiration and water absorption are restricted, probably by the presence of the inhibitor β complex. According to Hemberg [11, 12] this inhibitor suppresses the activity of enzymes containing thiol groups, and according to Marinos & Hemberg [15] it controls the amount of available ATP.

Since water absorption by potato tuber slices is dependent on respiration, it could be anticipated that addition of appropriate substrates would stimulate water uptake. The presented experiments concern the absorption of water by slices from resting tubers and tubers in post-dormant condition, in the presence of some metabolites of the tricarboxylic acid cycle.

MATERIAL AND METHODS

Potatoes "Dar" stored in a cellar at 6 - 8°, were used for experiments. Tubers of equal size were cut into 1 mm. thick slices with a microtome. Since in potatoes the metabolic reactions proceed mainly within the peripheral part of the tuber and inhibitor β is known to accumulate also in this part [10], disks 1 cm. in diameter were cut with a cork borer from each slice just within the peel and used for experiments. The disks were rinsed in running tap water for 30 min. and left in a small amount of water for 24 hr. at 10°. Then they were blotted with filter paper and weighed in groups of five on a torsion balance with an accuracy of 1 mg. This weight of 5 disks was called the initial fresh weight; it varied between 570 and 580 mg. Next, the respiration was determined and taken as the initial respiration value.

Each group of disks was then arranged on gauze stretched over Petri dishes, and placed in a crystallizer. The two vessels were filled with a tap-water solution of the compound to be studied; the pH was 6.6 and if necessary it was adjusted to this value with 0.1 N-NaOH or HCl. Then the crystallizer was placed in a thermostat at 25° and left in darkness. At two-day intervals the disks were weighed, submitted to respiration determinations, rinsed, and transferred to fresh solutions. Water absorption was determined as difference between the actual weight of the disks and their initial fresh weight. Each experiment was performed on ten parallel samples.

Respiration was measured by the direct Warburg method. The group of 5 disks was placed in a Warburg flask containing 1 ml. of 5 mM-phosphate buffer, pH 6.6; the central well contained 0.4 ml. of 20% KOH. Determinations of oxygen uptake were made at 25° during 2 hr. at 30 min. intervals. The respiration was calculated as $\mu l. O_2$ consumed by 5 disks during 30 min.

The following solutions were used: 0.01 mM-β-indolylacetic acid (auxin), 10 mM-glutathione, reduced (GSH), and 1 mM solutions of sodium salts of α-ketoglutaric, succinic, fumaric and malic acids. Auxin was a product of Merck (Germany); GSH and the dicarboxyl acids used were products of Fluka (Switzerland).

Standard error of the mean for the values presented in Table and Figures was calculated according to the formula:

$$\sqrt{\frac{\Sigma (\bar{x} - x)^2}{n (n - 1)}}$$

RESULTS

Water absorption and respiration of the dormant potato disks are shown in Fig. 1. Both α-ketoglutarate and malate inhibited water absorption (diagram *a*) but had no effect on oxygen uptake which attained a maximum after two days of incubation (diagram *b*). When, however, the disks were placed in a solution of α-ketoglutarate or malate which contained GSH or auxin (diagrams *d* and *f*, resp.), their respiration was enhanced and the inhibition of water absorption was not only overcome but this process was even enhanced (diagrams *c* and *e*).

The effect of GSH or auxin was most pronounced when they were added to malate. On the other hand, when α-ketoglutarate was applied as substrate the addition of GSH stimulated the respiration and overcame the inhibition of water absorption without enhancing it over the control values.

The endogenous respiration of disks was at a maximum after two days of incubation and then it began to decrease. After the addition of GSH and the substrates, the respiration also was the highest after two days and then slightly decreased; still, it remained high and did not fall below the control value. This seems to indicate that the substrate became incorporated in the respiratory metabolism of the disks from dormant tubers. GSH when applied alone did not stimulate water absorption but had a slightly activating effect on the respiration.

The effect of auxin was very similar to that of GSH. It should be noted, however,

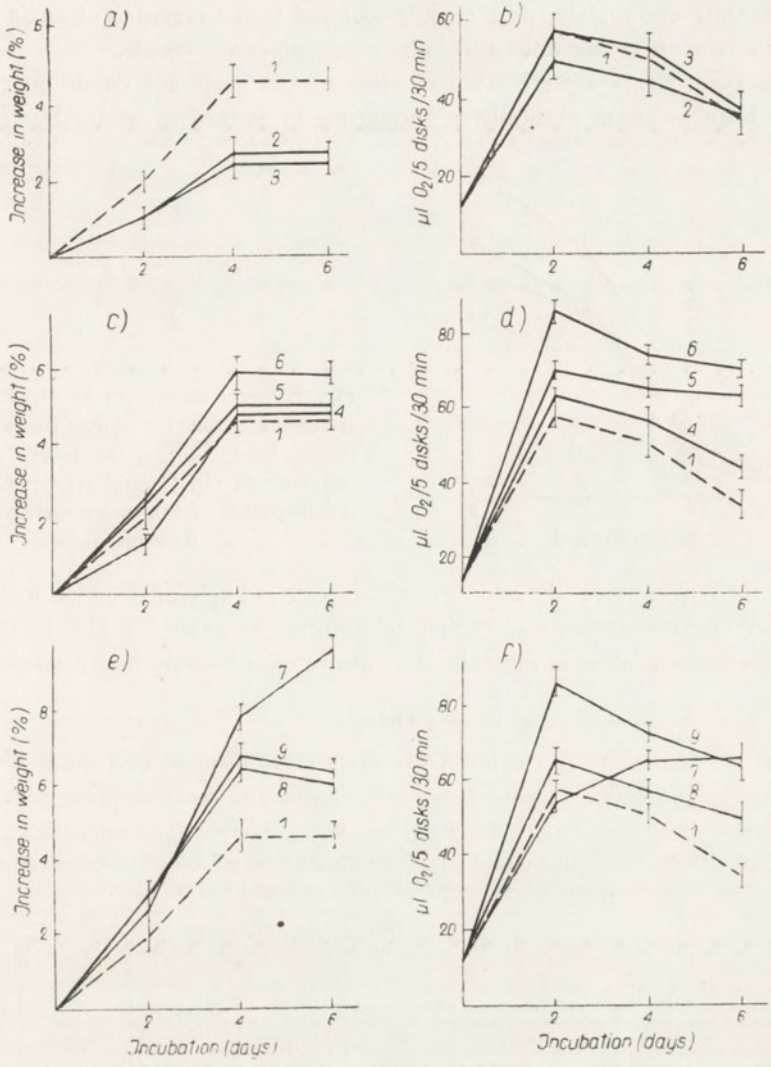

Fig. 1. Water absorption (diagrams *a*, *c*, *e*) and respiration (diagrams *b*, *d*, *f*) of disks from resting potato tubers. Conditions as described in the text. (*1*), Control (H_2O); (*2*), 1 mM-α-ketoglutarate; (*3*), 1 mM-malate; (*4*), 10 mM-glutathione; (*5*), 1 mM-α-ketoglutarate and 10 mM-GSH; (*6*), 1 mM-malate and 10 mM-GSH; (*7*), 0.01 mM-auxin; (*8*), 1 mM-α-ketoglutarate and 0.01 mM-auxin; (*9*), 1 mM-malate and 0.01 mM-auxin.

that the curves for the respiration in the presence of auxin alone differ from those for the metabolite of the tricarboxylic acid cycle and auxin added together. At first, that is after two days of incubation, the oxygen uptake in the presence of auxin alone was the same as for the controls but during the following days it increased gradually, whereas in the presence of both metabolite and auxin the respiration, after attaining a maximum after two days of incubation, diminished gradually. Probably in disks of resting potato tubers the respiratory substrates, such as

α-ketoglutarate and malate, were rapidly oxidized in the presence of auxin and the effect of auxin on endogenous respiration was no more visible.

When potato tubers were studied while in post-dormant condition (whether this was brought about naturally in springtime or evoked in winter by artificially

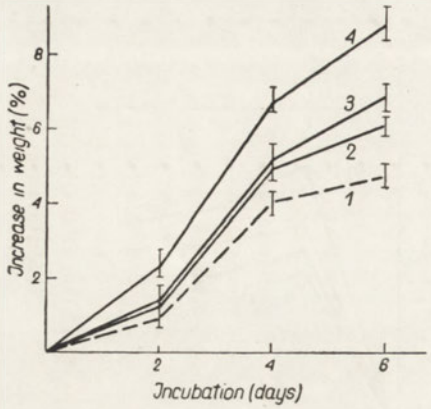

Fig. 2. Water absorption by disks from potatoes in which the dormancy was interrupted by submitting the tubers to a low temperature. (*1*), Control (H_2O); (*2*), 1 mM-α-ketoglutarate; (*3*), 1 mM-malate and (*4*), 0.01 mM-auxin.

lowering the temperature) the addition of substrate strongly affected the metabolism of disks. Water absorption was stimulated not only by auxin but also by the respiratory substrates (Table 1, Fig. 2). The substrates, however, had a weaker effect

Table 1

The effect of the metabolites of the Krebs cycle on respiration and water absorption by disks from potato tubers which emerged from dormancy

Oxygen uptake is expressed as μl. O_2/5 disks/30 min. Water absorption was determined as increase in weight. The results are mean values from 10 determinations ±S.D.E. The determinations were carried out between the 30th of April and 6th of May.

Addition	Day of determinations						
	0	2		4		6	
	Oxygen uptake	Oxygen uptake	Increase in weight (%)	Oxygen uptake	Increase in weight (%)	Oxygen uptake	Increase in weight (%)
Water (control)	24.8	50.1 ±2.68	4.5 ±0.73	45.7 ±5.35	6.9 ±0.49	39.2 ±4.12	7.2 ±0.60
α-Ketoglutarate, 1 mM	24.8	72.3 ±4.12	4.8 ±0.64	70.3 ±6.18	7.5 ±0.50	66.5 ±3.18	8.50 ±0.46
Malate, 1 mM	24.8	90.2 ±5.64	4.7 ±0.72	78.7 ±5.53	8.80 ±0.43	69.1 ±2.90	10.8 ±0.52
Succinate, 1 mM	24.8	85.6 ±3.18	4.9 ±0.81	82.7 ±4.96	7.6 ±0.61	77.4 ±3.12	9.5 ±0.55
Fumarate, 1 mM	24.8	93.4 ±2.73	4.9 ±0.89	71.9 ±5.18	9.4 ±0.59	62.2 ±4.37	8.8 ±0.52
Auxin, 0.01 mM	24.8	53.7 ±3.19	6.0 ±0.61	63.1 ±4.10	11.4 ±0.81	67.4 ±5.12	12.0 ±0.83

on the induced water absorption than auxin, malate being the most active and α-ketoglutarate the least active compound. Moreover, auxin stimulated water absorption already after two days of incubation (at the end of the lag phase) whereas it was only on subsequent days that the substrate-induced water absorption was markedly higher than in the controls.

The respiration of disks from tubers in post-dormant condition was strongly stimulated by the metabolites of the Krebs cycle. A maximum of oxygen uptake appeared after two days of incubation, similarly as in the controls, but it was higher by 40 - 80%, depending on the compound used. The respiration decreased with time but always remained higher than the respiration of controls.

The differences in auxin-induced and substrate-induced stimulation of respiration are clearly visible in Table 1. It appears that the metabolites of the Krebs cycle were rapidly incorporated into the respiratory processes, whereas the effect of auxin became manifest later, proceeding probably through a special mechanism.

DISCUSSION

The fact that the metabolites of the tricarboxylic acid cycle induce water absorption with simultaneous increase of respiration in slices of potato tubers in post-dormant condition, confirmed that water absorption is dependent on respiration. The lack of an inducing effect of the metabolites on respiration and water absorption in disks of dormant tubers indicates the presence of an inhibitor which suppresses the activity of the tricarboxylic acid cycle. This is probably the inhibitor β which, according to Hemberg [12], suppresses the activity of the thiol-group-containing enzymes; this inhibition is overcome by GSH [11, 12].

Resting tubers contain no GSH [9], therefore it seems justified to suppose that the addition of GSH activates the respiration system dependent on the tricarboxylic acid cycle, which in dormant tissue remains inactive. Probably the added GSH, by competing with the inhibitor, makes possible the oxidation of the metabolites. It is also possible that the system of electron transport postulated by Mapson & Goddard [14] is activated by the addition of GSH. This system, which includes NADP, GSH and ascorbic acid, can mediate the oxidation of metabolites of the Krebs cycle [3].

The effects of GSH and auxin (Fig. 1, diagrams *d* and *f*) on the oxidation of α-ketoglutarate and malate by resting potato tubers, are very similar indicating the activation of the same respiratory system, although probably through different mechanisms. Still, it is not to be excluded that auxin plays a role similar to that of GSH and activates some thiol-containing enzyme(s) despite the presence of the inhibitor β, but this possibility seems to be rather improbable.

It should be noted that auxin alone stimulated the respiration both in disks of dormant potato tubers and in disks from tubers in post-dormant condition. Since in either case the respiration and water absorption were simultaneously stimulated, it seems that auxin induces water absorption by enhancing respiration.

The possibility that auxin influences the respiration by taking part in the transport

of energy, has been discussed in the literature. In 1951 Bonner [1] suggested that auxin influences the synthesis of ATP [4]. Marinos & Hemberg [15] demonstrated that in resting potatoes the content of ATP is low, probably due to uncoupling of oxidative phosphorylation by the inhibitor β. The stimulation of ATP synthesis by auxin would explain its enhancing effect on the endogenous respiration of potato disks but it does not explain the effect of auxin on the exogenous respiration in the resting tubers.

The parallelism in activation of water absorption and respiration by GSH and auxin both in disks from resting potato tubers and from tubers in post-dormant condition points, as discussed above, to a direct or indirect participation of these compounds in ATP synthesis.

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SUMMARY

1. Incubation of disks from tubers which emerged from dormancy, with α-ketoglutarate, malate, succinate and fumarate stimulated oxygen uptake and water absorption, whereas during the resting period these compounds had no effect either on respiration or water uptake.

2. Addition of reduced glutathione or β-indolylacetic acid (auxin) together with the substrate enhanced the respiration in disks from resting tubers and simultaneously induced water absorption.

3. The possible influence of GSH and auxin on the metabolism of resting potato tubers is discussed.

REFERENCES

[1] Bonner J. - *Amer. J. Bot.* **36**, 429, 1949.
[2] Brauner L. & Hasman M. - *Protoplasma* **12**, 302, 1952.
[3] Eichenberger E. & Thimann K. V. - *Arch. Biochem. Biophys.* **67**, 466, 1957.
[4] French R. C. & Beevers H. - *Amer. J. Bot.* **40**, 660, 1953.
[5] Hackett D. P., Haas D. W., Griffiths S. K. & Niederpruen D. J. - *Plant Physiol.* **35**, 8, 1960.
[6] Hackett D. P., Schneiderman H. A. & Thimann K. V. - *Arch. Biochem. Biophys.* **47**, 205, 1953.
[7] Hackett D. P. & Thimann K. V. - *Amer. J. Bot.* **39**, 553, 1952.
[8] Hackett D. P. & Thimann K. V. - *Amer. J. Bot.* **40**, 183, 1953.
[9] Hemberg T. - *Physiol. Plant.* **3**, 17, 1950.
[10] Hemberg T. - *Physiol. Plant.* **7**, 312, 1954.
[11] Hemberg T. - *Physiol. Plant.* **11**, 615, 1958.
[12] Hemberg T. - *Physiol. Plant.* **14**, 861, 1961.
[13] Laties G. G. - *Plant Physiol.* **39**, 654, 1964.
[14] Mapson L. W. & Goddard D. R. - *Nature* **167**, 975, 1951.

[15] Marinos N. G. & Hemberg T. - *Physiol. Plant.* **13**, 571, 1960.
[16] Sperling E. & Laties G. G. - *Plant Physiol.* **38**, 546, 1963.
[17] Thimann K. V., Yocum C. S. & Hackett D. P. - *Arch. Biochem. Biophys.* **53**, 239, 1954.

ODDYCHANIE I POBIERANIE WODY W BULWACH ZIEMNIAKA PRZED I PO ZAKOŃCZENIU OKRESU SPOCZYNKU

Streszczenie

1. Inkubacja krążków ziemniaka po zakończeniu okresu spoczynku z α-ketoglutaranem, jabłczanem, bursztynianem lub fumaranem stymulowała równocześnie pobieranie wody i pobieranie tlenu; związki te nie miały natomiast wpływu na oddychanie i pobieranie wody u spoczynkujących ziemniaków.

2. Dodanie do substratu glutationu lub kwasu β-indolilo-octowego (auksyny) powodowało wzrost oddychania u spoczynkujących tkanek i równocześnie indukowało pobieranie wody.

3. Przedyskutowano możliwość wpływu GSH i auksyny na metabolizm spoczynkujących ziemniaków.

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HALINA AUGUSTYNIAK and J. AUGUSTYNIAK

THE NATURE OF THE BASIC COMPOUND FORMED FROM 2,5-DIKETOPIPERAZINE IN ACIDIC MEDIUM

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It has been observed [20] that a basic artifact containing glutamic acid and tyrosine, is formed during acid hydrolysis of γ-L-glutamyl-L-tyrosine. Some reactions suggested that this compound is of anhydride-type structure and its basic character indicated the presence of a structure enabling formation of a stable cation. A compound displaying similar properties has been also obtained from 2,5-diketopiperazine (DKP) and since it was found to be the simplest system of this type, it was used as model compound. On this model the following observations were made.

(1), The preparation of the "basic compound" [1] was obtained by saturating a suspension of DKP in dioxane (containing 2% of water) with gaseous HCl at room temperature, and it was separated from other reaction products by preparative electrophoresis or on a CM-Sephadex column using a buffer containing acetic acid. From 2 g. of DKP, 20 mg. of the "basic compound" acetate were obtained in which 1 molecule of acetic acid [2] corresponded to each atom of nitrogen.

None of the procedures applied caused splitting of the piperazine ring as the reduction of the "basic compound" by sodium borohydride or better by lithium aluminiumhydride resulted in formation of varying amounts of piperazine. Although some other reduction products were also obtained, it has been checked by electrophoresis and chromatography that similar products were formed also during the reduction of DKP. The reduction of DKP by sodium, as shown by Abderhalden *et al.* [4, 1] also leads to formation of piperazine and some other products.

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[1] The preparation of the "basic compound" submitted to high-voltage paper electrophoresis at pH 5.85 appeared to contain, in addition to a large amount of the "basic compound" whose mobility was similar to that of arginine, two other basic substances and a slight admixture of glycylglycine. All attempts at further purification were unsuccessful due to its unstability, therefore the mixture obtained was used for further determinations. Although the contaminating compounds did not exceed 10%, they precluded accurate quantitative determinations.

[2] When acetic acid was replaced by HCl, numerous products were obtained but no attempt was made to identify them.

(2), On acid hydrolysis the "basic compound" underwent decomposition with formation of glycine. When heated at 150° in water, it formed glycylglycine which on further heating was hydrolysed to glycine. After 8 hr. of heating, from 205 mg. of the "basic compound" acetate, 63.4 mg. of glycylglycine and 27 mg. of glycine were obtained.

(3), The "basic compound" acetate was rather stable in aqueous solution at pH 5.1, less stable at pH 1, and at pH about 10, similarly as DKP, immediately underwent decomposition with formation of glycylglycine.

(4), The "basic compound" gave a positive test with tetranitromethane, and with picric acid and hydroxylamine it gave colour reactions typical for anhydrides.

(5), An analogue of the "basic compound" was obtained from 3,6-dimethyl--DKP but numerous attempts at obtaining an analogue from 3,3,6,6-tetramethyl--DKP were unsuccessful. From *N,N'*-dimethyl-DKP only traces of a basic substance were formed.

(6), The infrared-absorption spectra of DKP and 3,6-dimethyl and 3,3,6,6--tetramethyl-DKP were similar and differed mainly in the bands corresponding to ν and β C—H present in CH_3 and CH_2 (near 3000 $cm.^{-1}$, 1380 $cm.^{-1}$ and 1150 $cm.^{-1}$). On the other hand, the spectrum of *N,N'*-dimethyl-DKP was markedly different. It did not contain the bands attributed by Katritzky [11] to the stretching modes of DKP ring vibrations; the absorption of ν C=O was shifted bathochromically by 10 - 30 $cm.^{-1}$ in relation to the absorption of ν C=O observed for the other anhydrides studied and of course no bands corresponding to free and associated ν N—H were obtained. The spectra of deuterated anhydrides (with the exception of *N,N'*-dimethyl-DKP) displayed a characteristic shift of the bands of ν N—H $\rightarrow$ ν N—D in agreement with the expected isotopic ratio. In deuterated DKP (Fig. 1) the absorption bands appeared in the region of 2480 - 2200 $cm.^{-1}$ (maximum at 2310 $cm.^{-1}$). Attempts at deuterium exchange in *N,N'*-dimethyl-DKP were unsuccessful; no difference in the spectra could be observed.

The infrared-absorption spectrum of the "basic compound" acetate (Fig. 3) in comparison with the spectrum of DKP showed the lack of strong absorption at 1690 $cm.^{-1}$ indicating total or partial disappearance of the carbonyl group; on the other hand, there appeared bands near 1560 and 1400 $cm.^{-1}$, and 1000 - 1100 $cm.^{-1}$ (C—O—C). In the region corresponding to ν O—H there was also a very strong and diffuse band near 3500 $cm.^{-1}$. The bands at 1560 and 1400 $cm.^{-1}$ originated from symmetric and asymmetric ν carboxyl anion; since they were missing from the spectrum of the perchloric salt, they appear to be connected with the presence of the acetate anion. The bands in the vicinity of 3500 and 1070 $cm.^{-1}$ indicate the presence of OH and C—O—C groupings.

(7), The preparation of the "basic compound" was electrophoretically compared with the "enol form" of DKP obtained by the method described by Abderhalden & Schwab [2, 3]. Spectrally, the "enol" of DKP appeared to be identical with standard DKP. Only in the crystallization mother liquor a substance was found whose electrophoretic properties were similar to those of the "basic compound". Therefore

it seems that the preparation studied by Abderhalden had a slight admixture of other compounds which could be responsible for the reducing properties and lower melting point of the supposed "enol form". Kellner [12] who spectrally studied the possibility of DKP-enolization did not mention the Abderhalden's "enol form"; probably she came to the same conclusion as the one presented above. Moreover, it was observed that DKP does not form polymorphic forms as the spectra of the preparations that had been crystallized from water, anhydrous ethanol or acetic acid, were identical over the range of 700 - 4000 $cm.^{-1}$.

These observations indicate that the "basic compound" studied is a peculiar ionic form of DKP displaying strong proton-acceptor properties manifested by the ability to give stable acetate and by marked electrophoretic mobility at pH 5.85. Formation of basic centres in a molecule of DKP may occur as result of disappearance of the carbonyl groups or of marked weakening of their influence on the adjacent nitrogen atoms.

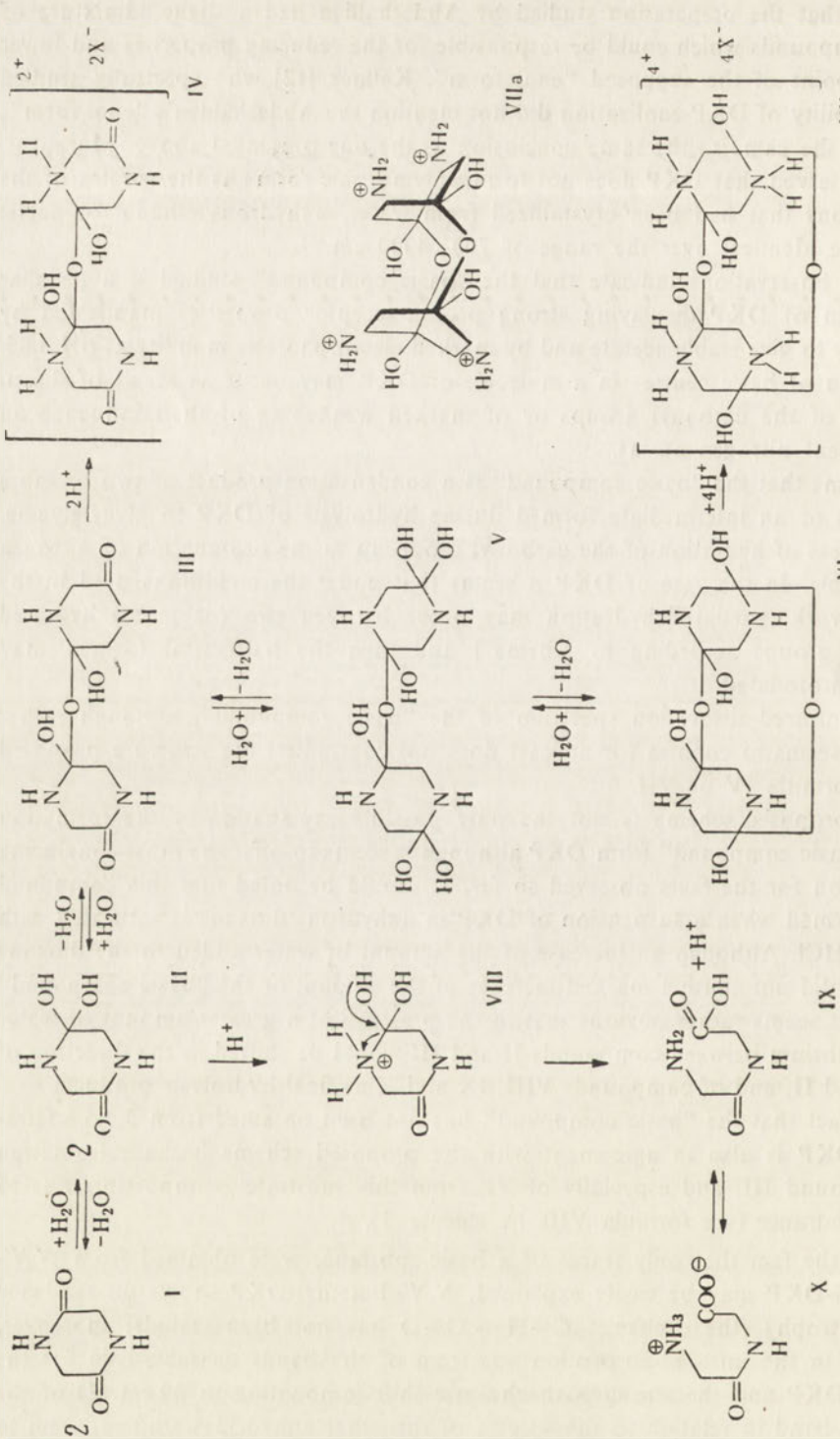
It seems that the "basic compound" is a condensation product of two or more molecules of an intermediate formed during hydrolysis of DKP to glycylglycine. The process of hydration of the carbonyl group up to the protonation of nitrogen is reversible. In the case of DKP it seems that under the conditions used in the present work, partial dehydration may occur between two (or more) hydrated carbonyl groups according to scheme 1 and then the half-acetal formed may become protonated.

The infrared-absorption spectrum of the "basic compound", although rather illegible, seems to confirm (or at least does not contradict) the structure proposed by the formula IV or VII.

The proposed scheme is not the only possible explanation of the formation of the "basic compound" from DKP although it seems to offer the most convincing explanation for the facts observed so far. It should be noted that this compound is not formed when a suspension of DKP in anhydrous dioxane is saturated with gaseous HCl. Although an increase of the amount of water added to the dioxane over 2% did not cause a marked increase of the amount of the "basic compound" formed, it seems rather obvious that in the presence of a greater amount of water the equilibrium between compounds II and III would be shifted in the direction of compound II, and of compounds VIII, IX and X as final hydrolysis products.

The fact that the "basic compound" has not been obtained from 3,3,6,6-tetramethyl-DKP is also in agreement with the proposed scheme because formation of compound III, and especially of VI, from this substrate is impossible, due to steric hindrance (see formula VIIa in scheme 1).

Also the fact that only traces of a basic substance were obtained from *N,N'*--dimethyl-DKP may be easily explained. *N,N'*-dimethyl-DKP shows no tendency to prototrophy (the exchange C—H → C—D has not been stated); moreover, the lack in the infrared-absorption spectrum of the bands characteristic for the ring of DKP and the strong bathochromic shift (amounting to 30 $cm.^{-1}$) of the carbonyl band in relation to the spectra of the other anhydrides studied, seem to suggest strong mesomerism which, however, is limited to the N—C=O grouping.



Scheme 1. The proposed formation of the "basic compound" from 2,5-diketopiperazine.

Probably, under such conditions the hydration of carbonyl might be hindered whereas protonation of nitrogen might be facilitated so that the formation of the "basic compound" analogue would be hampered or prevented whereas the hydrolysis would be facilitated. In addition the formation of a compound analogous to IV, and especially to VII, might be also prevented by steric hindrance.

From the proposed scheme it appears that compounds differing in their structure and condensation degree of molecules, might be formed from DKP. As previously mentioned, the preparation of the "basic compound" contained also some other substances which differed from the main compound probably by their degree of condensation. Quantitative prevalence of one of the compounds would indicate its privileged formation. However, the results obtained do not permit to establish its structure. So far, the suggested structure of this very unstable "basic compound" is but tentative and further data are required. Knowledge of the structure of the "basic compound" seems to be of importance since it is related to the basic artifacts formed during acid hydrolysis of many amino acids and might also bear some relation to the mechanism of hydrolysis of the peptide bond.

EXPERIMENTAL

Analytical methods. The melting points are uncorrected. Infrared-absorption spectra were recorded with a Perkin-Elmer Infracord 137 or UR-10 Zeiss spectrophotometer using the technique of suspension in paraffin oil (on average 5 mg. of the substance), or pressed KBr disks (about 1.7 mg. of substance per 300 mg. KBr), or as capillary film between plates of NaCl. High-voltage electrophoresis was performed after Masłowski [14] in concentrated pyridine buffer, pH 5.85, or 2 M-acetic acid. Paper chromatograms were developed in the following solvent systems: butan-1-ol - acetic acid - water (4:1:5, by vol.), butan-1-ol - acetic acid - water (4:1:1, by vol.) or pyridine - acetic acid - ethyl acetate - water (5:1:5:3, by vol.). The chromatograms were stained with ninhydrin, iodine vapour, or after Rydon & Smith [16].

For electrophoresis and CM-Sephadex column fractionation, pyridine - acetic acid buffers were used; the concentrated buffer consisted of 200 ml. of pyridine, 24 ml. of acetic acid, and water added to the final volume of 5 litres; the dilute buffer consisted of 1 vol. of the concentrated buffer and 4 vol. of water.

Preparation of anhydrides. 2,5-Diketopiperazine and glycylglycine were obtained by the method of Schott *et al.* [18]. The temperature of sublimation of DKP was 255 - 260° and the melting point 288°. The infrared-absorption spectrum of DKP is shown in Fig. 1a.

3,6-Dimethyl-2,5-diketopiperazine was prepared after Shibata & Asahina [19] or by heating DL-α-alanine with ethylene glycol according to Sannie [17]. Both preparations had the same melting point, 280°; the infrared spectrum is shown in Fig. 1b.

3,3,6,6-Tetramethyl-2,5-diketopiperazine was prepared initially from the methyl

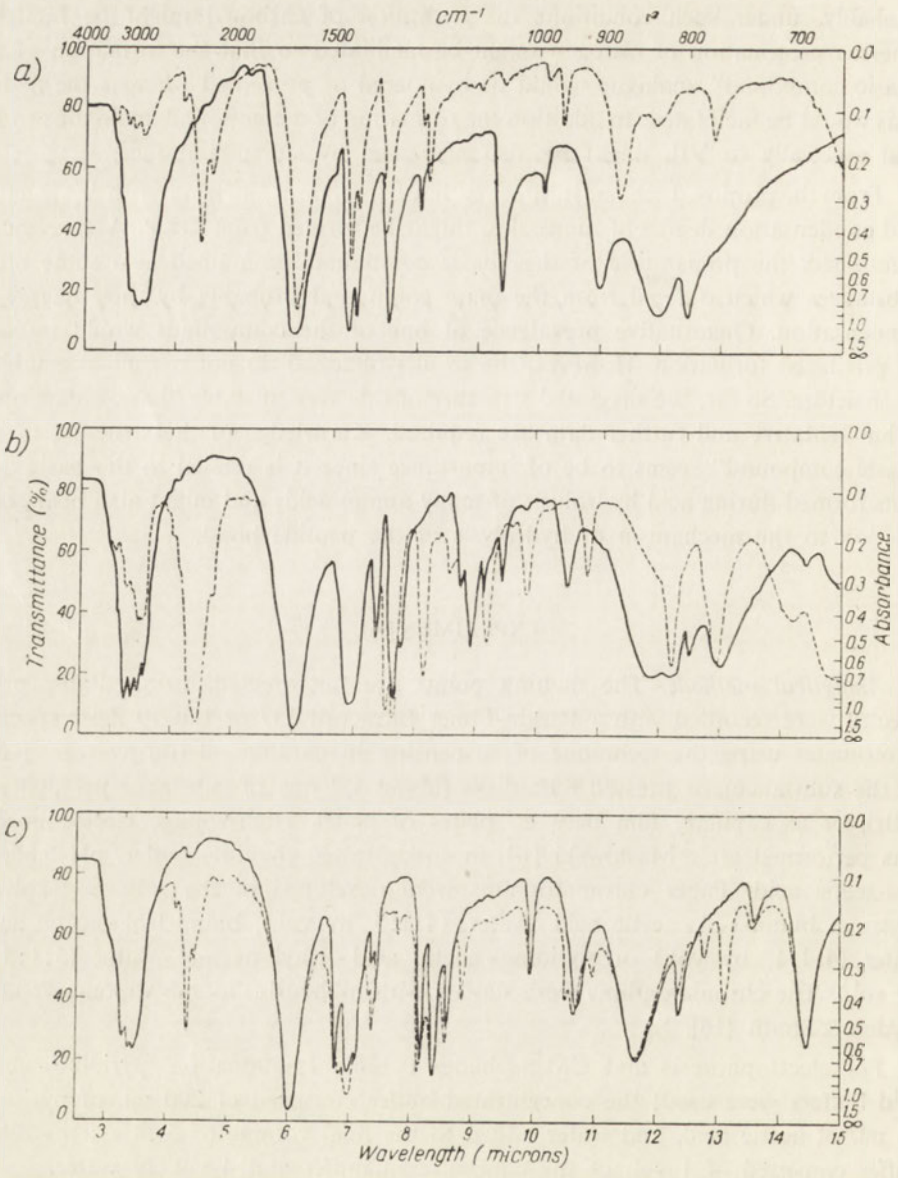


Fig. 1. Infrared-absorption spectra (KBr disks) of: (*a*), 2,5-diketopiperazine; (*b*), 3,6-dimethyl--2,5-diketopiperazine; (*c*), 3,3,6,6-tetramethyl-2,5-diketopiperazine. (—), Standards; (- - -), deuterated samples.

ester of α-aminoisobutyric acid according to Franchimont [10] but, as the yield was rather low, heating of 1 g. of DL-α-aminoisobutyric acid with 10 g. of ethylene glycol at 230° according to Sannie [17] was applied. The preparation was purified by crystallization from acetic acid; m.p. 355°; the infrared spectrum is shown in Fig. 1c.

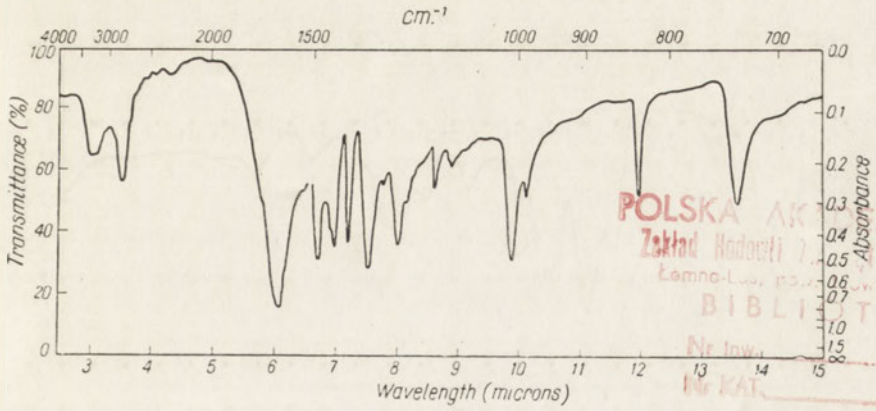


Fig. 2. Infrared-absorption spectrum of *N,N'*-dimethyl-2,5-diketopiperazine (KBr disk).

N,N'-Dimethyl-2,5-diketopiperazine was prepared according to Shibata & Asahina [19]; m.p. 147°; the infrared spectrum is shown in Fig. 2.

The anhydrides were deuterated in the following way: 15 mg. of the respective compound was added with 1.5 ml. of D_2O, the tube immediately sealed and heated at 80° for 48 hr. After 12 hr. at room temperature, the solvent was removed under reduced pressure and the residue was examined spectrally in compressed KBr disks.

Preparation of the "basic compound"

The following procedure was adopted: 2 g. of DKP were suspended in 180 ml. of dioxane containing 2% of water, then the mixture was saturated with gaseous HCl for 2 hr. with constant stirring. The suspension was filtered and the filtrate was evaporated on a rotary vacuum evaporator at 40°. The residue was added with about 5 ml. of anhydrous methanol, the solution transferred to a centrifuge tube and methanol was evaporated with a stream of air. The precipitate of DKP was centrifuged off and the oil (about 460 mg.) containing mainly the "basic compound" and glycylglycine was fractionated on a CM-Sephadex C-25 medium column (23×1.4 cm.) which had been equilibrated with dilute pyridine - acetic acid buffer, pH 5.85. The column was eluted first with 150 ml. of the same buffer, then with 350 ml. of the concentrated buffer, 7-ml. fractions being collected. Neutral substances (mainly glycylglycine) were eluted with the diluted buffer, and the "basic compound" with the concentrated buffer. The fractions containing the "basic compound" were pooled, evaporated on a rotary vacuum evaporator and rechromatographed as described above.

The collected eluates of the basic fraction were additionally purified in an 8-compartment apparatus for preparative electrophoresis [5]. Five ml. of the concentrated pyridine - acetic acid buffer containing about 40 mg. of the substance, were placed in the anodic compartment. Other compartments contained each 5 ml. of the same buffer. After 1 hr. separation at 220 V and 100 mA, the pooled contents of the cathodic

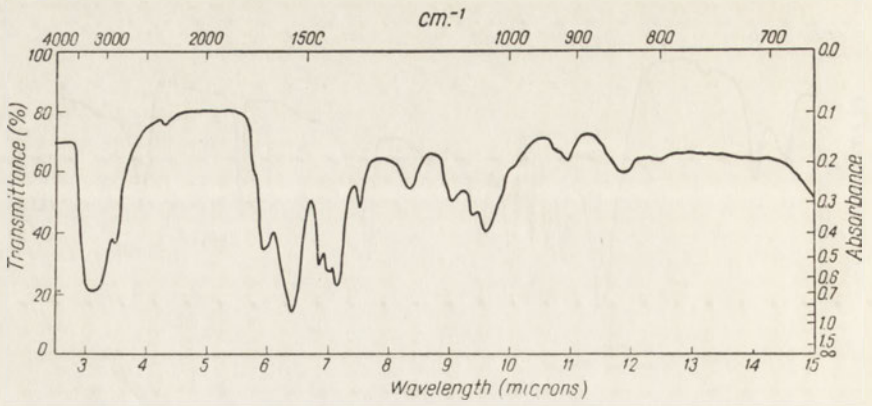


Fig. 3. Infrared-absorption spectrum of acetate of the "basic compound" obtained from 2,5-diketopiperazine (KBr disk).

compartments 7 and 8 were evaporated on a rotary vacuum evaporator to the volume of 5 ml. and submitted twice to electrophoresis under the same conditions. After the third separation, the substances from compartments 7 and 8 were dried over P_2O_5 and used for further studies.

By the procedures described, from 2 g. of DKP 20 mg. of the "basic compound" were obtained. The infrared spectrum of the preparation is shown in Fig. 3.

Attempts to obtain analogues of the "basic compound" from 3,6-dimethyl-, 3,3,6,6-tetramethyl-, and *N,N'*-dimethyl-DKP were made by saturating with gaseous HCl a suspension of the respective anhydride in dioxane containing 2% of water. After 2 hr. the suspension was filtered, the dioxane evaporated and the residue examined by electrophoresis and paper chromatography.

Properties of the "basic compound"

Reduction. Ten mg. of the preparation were dissolved in a small amount of water and added with 10 mg. of sodium borohydride. The mixture was heated for 15 min. at 80°, acidified with 0.5 N-HCl and left for 2 hr. Then the solution was alkalized with KOH and steam-distilled for 15 min. The distillate was acidified, evaporated to dryness and submitted to paper electrophoresis and chromatography.

The reduction with lithium aluminiumhydride was carried out by the methods of Nystrom & Berger [15], Dukas & Fontaine [8], and Langebeck *et al.* [13]. Satisfactory results were obtained by the method of Langebeck *et al.* From 30 mg. of the preparation 6.3 mg. of hydrochlorides of the steam-volatile substances were obtained; piperazine was the main compound as shown by chromatography and paper electrophoresis as well as by the infrared spectra of its hydrochloride or perchlorate. Before infrared-absorption analysis the samples were treated with charcoal.

The catalytic reduction in the presence of platinum according to Adams or to Brown & Brown [7] did not lead to formation of piperazine.

Acid hydrolysis. The "basic compound" was heated in sealed tubes in hydrochloric acid of constant boiling point for 24 hr. at 100°, 0.2 ml. of the acid being added per 1 mg. After hydrolysis, the excess of the acid was removed by evaporation under reduced pressure at 40°. Among the hydrolysis products, glycine was identified by chromatography and paper electrophoresis.

Water hydrolysis. Water solution of the "basic compound" at a concentration of 15 mg./ml. was heated in sealed tubes at 150° for 1, 6, 12, 24 and 48 hr. Then the hydrolysis products were submitted to paper electrophoresis in 2 M-acetic acid. From Fig. 4 it appears that on prolonged hydrolysis the "basic compound" becomes gradually converted to glycine with glycylglycine as an intermediate product.

To check the electrophoretic observations, 14.8 mg. of the "basic compound" were dissolved in 1 ml. of water and heated for 62 hr. at 150°. The hydrolysate was separated on CM-Sephadex C-25 column (10×1.2 cm.) in the same way as

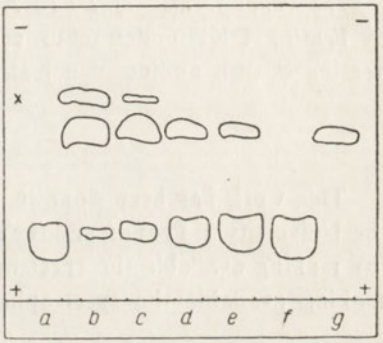


Fig. 4. Electrophoretogram: (*a*), glycine standard; (*g*), glycyl-glycine standard; *b* to *f*, water hydrolysate of the "basic compound". Time of hydrolysis of the respective samples: *b*, 1 hr.; *c*, 6 hr.; *d*, 12 hr.; *e*, 24 hr.; *f*, 48 hr. Conditions of electrophoresis: 2 M-acetic acid, voltage 3 KV, time of separation 1.5 hr. The samples *b* and *c* contain visible amounts of the "basic compound" (x).

the "basic compound", 2-ml. fractions being collected. In the fractions 2 - 10, 8.4 mg. of a compound were obtained whose infrared-absorption spectrum was identical with that of glycine.

Quantitative determination of glycine and glycylglycine. The "basic compound" was submitted to water hydrolysis for 8 hr. and the hydrolysate was treated with 1-fluoro-2,4-dinitrobenzene using the procedure 1 of Fraenkel-Conrat *et al.* [9]. The DNP-compounds were extracted with ethyl acetate and the extract was chromatographed in butan-1-ol saturated with 1% aqueous ammonia [6]. Standard mixtures of glycine and glycylglycine were treated with dinitrophenyl and chromatographed in the same way. The spots were eluted and in the eluates the extinctions at 360 mμ were examined. The amounts of glycine and glycylglycine in the hydrolysate were determined by comparing their extinctions with those of the similarly treated standard solutions.

The content of acetate in the "basic compound". A known amount of the "basic compound" was acidified with 12 N-H_2SO_4 and steam-distilled for 15 min. The distillate was titrated potentiometrically with 0.02 N-NaOH and the amount of the

acetic acid calculated. Then the neutralized distillate was evaporated to dryness and its infrared spectrum was found to be identical with that of sodium acetate.

The behaviour of solution of the "basic compound" at different pH *values.* A solution of 37 mg. of the "basic compound" in 5 ml. of water was divided into three portions. One of them was brought to pH 1 by adding HCl, the second to pH 10 by adding NaOH, and the third one, untreated, had a pH of 5.1. All three solutions were kept at room temperature and from time to time samples were submitted to paper electrophoresis in 2 M-acetic acid. After 42 days of storage, in the water solution a large amount of the "basic compound" was found and a small amount of glycylglycine and glycine; in the acidic solution the amount of the "basic compound" was small and that of glycylglycine and glycine was large, whereas in the alkaline solution immediately after NaOH addition glycylglycine, and after 42 days also much of glycine was found. When the solutions were kept at 40°, the hydrolysis was greater, and the "basic compound" present in smaller amounts. The amounts of the compounds formed were evaluated only approximately by comparing the electrophoretograms of solutions stored for varying periods of time.

The "enol forms" of DKP. Attempts to obtain these compounds were made by heating DKP under reflux according to Abderhalden & Schwab [2, 3] or by heating it with aniline in a sealed tube.

This work has been done in part in the Department of Organic Chemistry of the University in Poznań and the authors express their thanks to Prof. Dr. J. Suszko for making available the spectrophotometer UR-10 and to Prof. Dr. J. Bartz for making available the spectrophotometer Perkin-Elmer Infracord 137.

SUMMARY

By saturating with gaseous HCl a suspension of 2,5-diketopiperazine in dioxane containing 2% of water, a basic compound was obtained. Reduction to piperazine, hydrolysis to glycylglycine and glycine, infrared-absorption spectrum, and decomposition in alkaline medium indicate that this compound is a condensation product of two or more molecules of an intermediate formed during hydrolysis of 2,5-diketopiperazine to glycylglycine. It is suggested that the basic compound formed from amino acids on acid hydrolysis may have a similar structure.

REFERENCES

[1] Abderhalden E. & Schwab E. - *Z Physiol. Chem.* **139**, 68, 1924.
[2] Abderhalden E. & Schwab E. - *Z. Physiol. Chem.* **149**, 100, 1925.
[3] Abderhalden E. & Schwab E. - *Z. Physiol. Chem.* **153**, 83, 1926.
[4] Abderhalden E., Klarman E. & Schwab E. - *Z. Physiol. Chem.* **135**, 180, 1924.
[5] Augustyniak J. & Augustyniak H., *I Ogólnopolskie Sympozjum z Elektroforezy, Gdańsk* 1964, p. 31.

[6] Braunitzer G. - *Chem. Ber.* **88**, 2025, 1955.
[7] Brown Ch. A. & Brown H. C. - *J. Amer. Chem. Soc.* **84**, 2827, 1962.
[8] Dukas H. M. & Fontaine T. D. - *J. Amer. Chem. Soc.* **73**, 5917, 1951.
[9] Fraenkel-Conrat H., Harris J. I. & Levy A. L. - *Methods of Biochem. Anal.* **2**, 370, 1955.
[10] Franchimont M. M. A. P. N. & Friedmann H. - *Rec. Trav. Chim. Pays-Bas* **27**, 197, 1908.
[11] Katritzky A. R., *Physical Methods in Heterocyclic Chemistry*, vol. II, p. 272. Academic Press, New York 1963.
[12] Kellner L. - *Nature* **140**, 193, 1937.
[13] Langebeck V., Augustin M., Böhm R. & Hoffmann S. - *J. Prakt. Chem.* **25**, 301, 1964.
[14] Masłowski P. - *Chem. Anal.* **4**, 611, 1959.
[15] Nystrom R. F. & Berger C. R. A. - *J. Amer. Chem. Soc.* 2896, 1958.
[16] Rydon H. N. & Smith P. W. G. - *Nature* **169**, 922, 1952.
[17] Sannie C. - *Bull. Soc. Chim. France* **9**, 487, 1942.
[18] Schott H. F., Larkin I. B., Rockland L. B. & Dunn M. S. - *J. Org. Chem.* **12**, 490, 1847.
[19] Shibata Y. & Asahina T. - *Bull. Chem. Soc. Japan* **1**, 71, 1926; cited after C 1926 II p. 219.
[20] Wiewiórowski M. & Augustyniakowa H. - *Acta Biochim. Polon.* **9**, 399, 1962.

O BUDOWIE ZASADOWEGO PRODUKTU POWSTAJĄCEGO Z 2,5-DWUKETOPIPERAZYNY W KWAŚNYM ŚRODOWISKU

Streszczenie

Z 2,5-dwuketopiperazyny otrzymano zasadowy związek wysycając gazowym HCl jej zawiesinę w dioksanie zawierającym 2% wody. Na podstawie dokonanych obserwacji (redukcja związku do piperazyny, hydroliza prowadząca do glicylo-glicyny i glicyny, widmo IR, szybki rozpad w zasadowym środowisku) przypuszcza się, że związek ten powstaje w wyniku kondensacji dwu lub więcej pośrednich produktów tworzących się na drodze hydrolizy 2,5-dwuketopiperazyny do glicylo-glicyny. Sugeruje się, że zasadowe związki powstające z aminokwasów w warunkach kwaśnej hydrolizy mają zbliżoną strukturę.

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TEMPERATURE DEPENDENCE OF AMP DEAMINATION CATALYSED BY MUSCLE EXTRACTS FROM HOMEOTHERMIC AND POIKILOTHERMIC ANIMALS

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Few experiments have been described concerning the temperature dependence of the enzymes catalysing the same reaction in homeothermic and poikilothermic animals [6, 7], and usually in the experiments the enzyme was saturated with substrate and hence the maximum velocity was measured. However, the conditions prevailing in the cell may differ widely from those needed to achieve the maximum reaction rate. Actually the substrate concentration in the living cell may be so low that the reaction would follow the first-order kinetics in respect to substrate.

In this paper temperature dependence of the rate of reaction catalysed by AMP aminohydrolase (EC 3.4.5.6) present in the muscles of homeothermic and poikilothermic animals was investigated at very low substrate concentration. Under these conditions the temperature dependence of the enzymic reaction seems to be more compatible with the physiological behaviour of both animal groups than at high substrate concentrations.

MATERIALS AND METHODS

The animals used were either laboratory-bred (rabbit, rat), obtained from commercial sources (pigeon, trout), or they were caught in their natural environment (frog, carp). The animals were killed by decapitation, skeletal muscle was taken as soon as possible and a 10% homogenate in 0.6 M-KCl was prepared in a Potter-Elvehjem type homogenizer chilled with ice. After the homogenization had been continued for 5 min., the homogenate was centrifuged at 25 000 *g* for 15 min., and the resulting supernatant called muscle extract was used for the experiments.

The reaction rates were measured in a system containing 0.1 M-sodium succinate buffer, pH 6.6, 0.6 M-KCl, appropriate concentration of AMP, and an amount of muscle extract depending on the specific activity of a particular muscle (usually it was the amount corresponding to 1.5 - 6.0 mg. of fresh muscle per sample). When very low substrate concentrations were used and hence the reaction kinetics could

be followed spectrophotometrically according to Kalckar [3], the incubation was carried out in a Unicam SP-500 spectrophotometer fitted with a constant temperature cell housing. In this case the readings at 265 mμ were taken every one or two minutes until about half of the substrate was decomposed and mean value of the first-order rate constant k was calculated from all the readings. In experiments in which high substrate concentration was used, the incubation was performed in test tubes and ammonia liberated was the measure of the reaction rate. Ammonia was estimated with the Nessler reagent according to Bock & Benedict [2].

RESULTS

Muscle extracts from three homeothermic animal species: rabbit (*Oryctolagus cuniculus*), rat (*Rattus norvegicus*) and pigeon (*Columba livia*) and from three poikilotherms: frog (*Rana esculenta*), carp (*Cyprinus carpio*) and trout (*Salmo irideus*) have been investigated. When the reaction rate of AMP deamination was measured at very low, 54 μM, substrate concentration at ten temperature levels ranging from

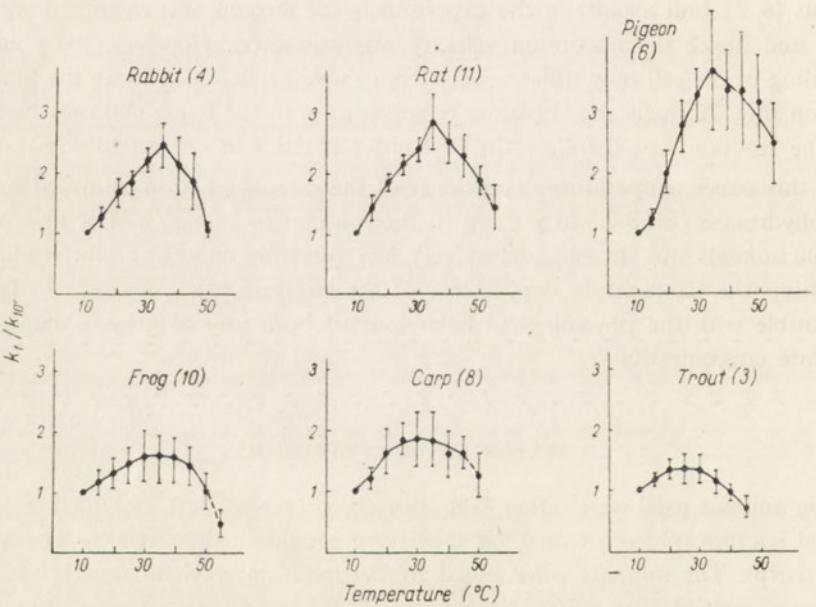

Fig. 1. The influence of temperature on the rate of AMP deamination catalysed by muscle extracts from poikilothermic and homeothermic animals at very low substrate concentration (54 μM). Incubation was carried out in 0.1 M-sodium succinate buffer, pH 6.6, containing 0.6 M-KCl. The reaction was initiated by adding 20 μl. of muscle extract to 3 ml. of the substrate solution. The readings of extinction at 265 mμ were taken every 1 or 2 min. with a Unicam SP-500 spectrophotometer fitted with a constant temperature cell housing. The values on the ordinate represent the ratio of the first-order rate constant at a given temperature k_t to the k at 10°, i.e. the figures indicate how many times the rate at a given temperature increased as compared with the rate at 10°. Mean values are presented ±S.D. The number of animals investigated is given in parentheses.

10° to 55°, wide differences were observed between the poikilothermic and homeothermic animals. Whereas a sharp temperature optimum at 35° was found for the three homeothermic animals, no optimum at all could be observed for the poikilotherms (Fig. 1). The rate of the reaction catalysed by trout muscle extract did not change between 20° and 30°; the same was found for the carp between 25° and 35° and for the frog between 30° and 40°. In all poikilotherms studied, also at other temperatures investigated the reaction rate hardly differed from that at 10°. In homeothermic animals, pigeon, rat and rabbit, the rate of the reaction catalysed by muscle extracts increased by a factor of 2.5 - 4 when the temperature was raised from 10° to 35°. Above 35° the rate decreased, although at 40°, 45° and even at 50° the reaction was still of the first-order. It means that at these temperatures no other factor than substrate concentration influenced the reaction rate while the measurements were continued.

As the measurements have been done at temperature intervals of 5° one could suppose that the optimum temperature for the homeothermic animals enzyme is slightly different from 35°, or that the temperature optimum for the poikilothermic animals enzyme has been overlooked. The experiments presented in Table 1 indicate that this was not the case. AMP aminohydrolase from rat muscle showed a rather

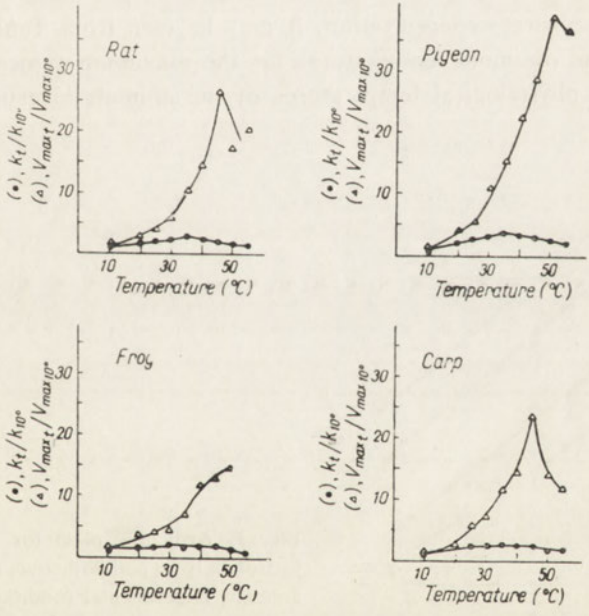

Fig. 2. The influence of temperature on the changes of maximum velocity (V_{max}) and on the first-order reaction rate constant (k). To estimate V_{max} an appropriate amount of muscle extract was incubated with different concentrations of AMP dissolved in 0.1 M-sodium succinate buffer, pH 6.6, containing 0.6 M-KCl; after the incubation mixture had been deproteinized with trichloroacetic acid, ammonia was estimated by nesslerization and V_{max} calculated from the Lineweaver-Burk plot. For measuring the first-order rate constant k, the incubation conditions were the same except that substrate concentration was much lower (54 μM); the reaction was followed spectrophotometrically as described in Fig. 1.

Table 1

The influence of temperatures 31° - 43° on AMP deamination catalysed by frog and rat muscle extracts

Experimental conditions as in Fig. 1. The figures represent mean values of the ratio of the rate constant k at a given temperature to the rate constant at 10°±S.D. In parentheses the number of animals is given.

Animal	$\frac{k_{31°}}{k_{10°}}$	$\frac{k_{33°}}{k_{10°}}$	$\frac{k_{35°}}{k_{10°}}$	$\frac{k_{37°}}{k_{10°}}$	$\frac{k_{39°}}{k_{10°}}$	$\frac{k_{41°}}{k_{10°}}$	$\frac{k_{43°}}{k_{10°}}$
Frog (3)	1.89±0.16	1.71±0.33	1.62±0.75	1.68±0.21	1.68±0.16	1.47±0.48	1.55±0.16
Rat (3)	2.80±0.56	2.91±0.75	3.18±0.72	3.04±0.71	2.55±0.43	2.35±0.44	2.27±0.46

sharp optimum at 35° whereas the rate of the reaction catalysed by frog muscle extract changed little at any temperature between 31° and 43°.

High substrate concentrations were used for estimations of V_{max} for the reactions catalysed by muscle extracts from the frog, carp, rat and pigeon. It appeared that temperature-dependent changes in V_{max} are different from those observed for the velocity at low substrate concentration. It may be seen from Table 2 and Fig. 2 that not only the optimum temperatures for the maximum velocities were much higher than the physiological temperatures of the animals investigated, but also

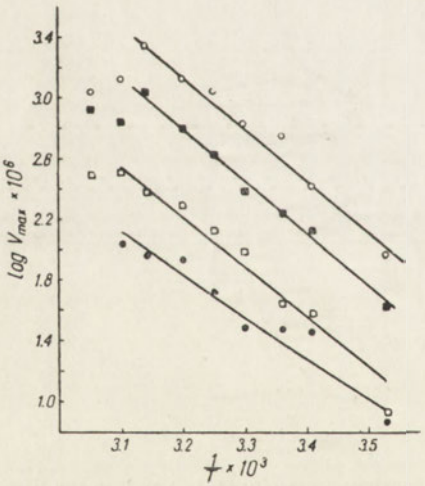

Fig. 3. Arrhenius plots for the AMP aminohydrolase from poikilothermic and homeothermic animals. Experimental conditions as in Fig. 2. (●), Frog; (○), carp; (■), rat, (□), pigeon

the increase of the maximum rate caused by the change of temperature was about 10 times higher than the increase of the rate at low substrate concentration both in poikilothermic and homeothermic animals. When log V_{max} has been plotted against reciprocal of the absolute temperature (Fig. 3) for all reactions, independently of the source of the enzyme, the slopes were very similar; hence it appears that the

Table 2

The influence of temperature on maximum velocity (V_{max}) *and on the first-order reaction constant* (k) *in poikilothermic and homeothermic animals*

The values of V_{max} are presented as M/min.; a 2 ml. sample contained the amount of extract corresponding to 1.6 mg. of muscle. The first-order rate constant k was estimated by using the same muscle extract. The values of k are presented as $min.^{-1}$ for the same enzyme concentration as in the samples in which V_{max} was estimated. For experimental conditions see Fig. 2.

Animal		10°	20°	25°	30°	35°	40°	45°	50°	55°
Frog	$V_{max} \times 10^6$	7.5	29	30	31	52	88	94	110	
	k	0.0168	0.0181	0.0231	0.0265	0.0239	0.0246	0.0261	0.0181	0.0092
Carp	$V_{max} \times 10^6$	92	260	550	660	1100	1300	2200	1300	1100
	k	0.0927	0.2320	0.2250	0.1950	0.2290	0.2170	0.1910	0.1390	0.1550
Rat	$V_{max} \times 10^6$	41	130	170	240	420	600	1100	700	840
	k	0.0490	0.0872	0.0894	0.1064	0.1302	0.1121	0.1007	0.0775	0.0759
Pigeon	$V_{max} \times 10^6$	8.2	37	44	96	130	190	240	320	310
	k	0.0273	0.0528	0.0746	0.0816	0.0985	0.0895	0.0855	0.0779	0.0692

activation energy was the same in both animal groups. This is at variance with the experiments of Vroman & Brown [7] who found a considerably lower activation energy for succinic dehydrogenase obtained from frog liver than for the same enzyme from rat liver.

DISCUSSION

The results presented indicate that in animals having an efficient thermoregulation system, at low substrate concentration the optimum temperature for muscle AMP aminohydrolase is at 35°. In poikilothermic animals which undergo considerable temperature variations during their life, the changes of temperature over a wide range cause little, if any, change in the reaction rate. This is valid only for the conditions under which the enzyme is not saturated with substrate and the reaction is first-order:

$$E+S \rightleftharpoons ES \rightarrow E+P \qquad (1)$$

At low substrate concentration the reaction rate depends mainly on the first reaction of the equation (1) i.e. on the binding of the enzyme with substrate. This reaction might be characterized by the substrate constant K_s. It would mean that in poikilothermic animals appropriate variation of K_s with temperature is the factor responsible for the invariability of the rate constant k over a wide range of temperatures. However, the reaction of the decomposition of the enzyme - substrate complex into enzyme and product is strongly accelerated by the increase of temperature as indicated by rapid changes of V_{max} both in poikilothermic and homeothermic animals.

It may be shown, using the integrated Michaelis-Menten equation, that at very low substrate concentration the enzymic reaction has to be first-order [1] and an interrelationship may be derived for these conditions between the rate constant k and V_{max}:

$$k = \frac{V_{max}}{K_m} \qquad (2)$$

We have shown in our experiments that the reaction catalysed by muscle AMP aminohydrolase was first-order at substrate concentration of 54 μM. At this substrate concentration the dependence of the rate constant k on temperature was different in homeothermic and poikilothermic animals. Whereas in homeotherms the changes of temperature affected strongly the k value, with poikilothermic animals enzyme large variations of temperature caused insignificant changes of the reaction rate. As V_{max} changed with temperature in a similar way both in poikilothermic and homeothermic animals, it is most probable that the temperature influences in a different way K_m in homeothermic and in poikilothermic animals. To prove this it would be necessary to investigate the variation of K_m with temperature both in poikilothermic and homeothermic animals.

The consistency of the temperature dependence of k with the physiological behaviour of the temperature of the whole animal's body allows to suppose that some adaptation to the presence or to the lack of thermoregulation exists also at the enzymic level. This conclusion is in accordance with the recent findings of Licht [4, 5] who showed that in some species of lizards the temperature dependence of muscle ATPase and of isometric muscle twitch is consistent with the "preferred temperature" of these animals.

The authors appreciate skilled technical assistance of Mrs. Melania Łabuda.

SUMMARY

The temperature dependence of the rate of AMP deamination has been measured at the substrate concentration 54 μM at which the reaction is first-order. For muscle extracts of three homeothermic animals, a sharp temperature optimum was observed at 35°. With the enzyme from any poikilothermic animal there was no sharp temperature optimum and the reaction rate did not increase more than twice at any higher temperature as compared with the rate at 10°.

The maximum reaction rate increased 7 - 16 times with the change of temperature from 10° to 35° in both animal groups.

REFERENCES

[1] Dixon M. & Webb E. C., *Enzymes*, p. 119; Longmans, Green & Co., London, New York, Toronto 1958.
[2] Hawk P. B., Oser B. L. & Summerson W. H., *Practical Physiological Chemistry*, p. 1230; The Blakiston Company, Philadelphia, Toronto 1949.
[3] Kalckar H. M. - *J. Biol. Chem.* **167**, 461, 1947.
[4] Licht P. - *Comp. Biochem. Physiol.* **12**, 331, 1964.
[5] Licht P. - *Comp. Biochem. Physiol.* **13**, 27, 1964.
[6] Sizer I. W. - *Advanc. Enzymol.* **3**, 35, 1943.
[7] Vroman H. E. & Brown J. R. C. - *J. Cell. Comp. Physiol.* **61**, 129, 1963.

WPŁYW TEMPERATURY NA DEZAMINACJĘ AMP KATALIZOWANĄ PRZEZ WYCIĄGI MIĘŚNIOWE ZWIERZĄT STAŁOCIEPLNYCH I ZMIENNOCIEPLNYCH

Streszczenie

Badano wpływ temperatury na szybkość dezaminacji AMP w stężeniu substratu równym 54 μM, w którym reakcja przebiega według kinetyki pierwszego rzędu. Używając wyciągów mięśniowych trzech zwierząt stałocieplnych (szczur, królik i gołąb) zaobserwowano ostre optimum temperatury przy 35°. Dla wyciągów z mięśni zwierząt zmiennocieplnych (żaba, pstrąg i karp) nie zaobserwowano takiego optimum, a szybkość reakcji w każdej wyższej temperaturze nie przekraczała dwukrotnej szybkości przy 10°.

V_{max} wzrastało 7 - 16 razy przy wzroście temperatury od 10° do 35° dla obu grup zwierząt.

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SOME PROPERTIES OF ELASTIN DEGRADATION PRODUCTS

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Elastin, the insoluble protein which is the main constituent of elastic tissue, can be brought into solution by treatment with acid or alkali or by some proteolytic enzymes. The dissolution is of hydrolytic nature and products of different size are formed.

Among the degradation products two soluble proteins were isolated by Partridge *et al.* [7], one of relatively high molecular weight (60 000 - 84 000), α-elastin, and another of low molecular weight (about 5500), β-elastin. The amino acid composition of these two proteins is almost the same and does not differ from that of the insoluble elastin [8]. The main property distinguishing the two soluble proteins is the coacervation of α-elastin in solution during heating at pH values ranging from 4 to 6. Under these conditions the solution becomes opaque and liquid droplets consisting of a highly viscous material are formed [1, 7, 4].

The purpose of the experiments described in this paper was to study some properties of the separated degradation products of elastin which would enable to distinguish them in mixture and facilitate their isolation, and which could be utilized as well for studying the kinetics of elastolysis.

MATERIALS AND METHODS

Preparation of elastin. Elastin was obtained from ox ligamentum nuchae according to Partridge *et al.* [7]. The preparation was dehydrated with ethanol and dried to constant weight over P_2O_5 under reduced pressure at 80°.

Dissolution of elastin. This was performed by boiling with 0.25 M-oxalic acid according to Partridge *et al.* [7]. Oxalic acid was removed by adding finely powdered calcium carbonate with constant stirring; after neutralization, the stirring was continued for 60 min. The mixture was left overnight at 0°, then centrifuged at 5000 rev./min. for 15 min. and exhaustively dialysed against water.

Separation of α- and β-elastin. The clear, dark-yellow solution obtained after dialysis was adjusted to pH 4.7 and to ionic strength of 0.01 with 4 M-acetate buffer

according to Partridge *et al.* [7]. A coacervate which formed on mild heating to 50° and gentle stirring was collected easily and completely on a glass rod, and dissolved in cold acetate buffer; it was found to contain mainly α-elastin. The protein remaining in the solution after coacervation consisted mainly of β-elastin. Further purification was performed according to Partridge *et al.* [7]. Solutions of α- and β-elastin, after being dialysed against water, were freeze-dried and the preparations stored at −20°. Some analytical data for α-elastin and β-elastin, together with those for the starting preparation of the insoluble elastin, are presented in Table 1.

Table 1

Some analytical data for insoluble and soluble elastin preparations obtained from ox ligamentum nuchae

Preparation	Nitrogen (%)	Hexosamine (%)	Uronic acid (%)
Insoluble elastin	16.50	0.44	0.10
Insoluble elastin	16.43	0.25	0.08
α-Elastin	15.85	0.30	0.09
α-Elastin	15.71	0.33	0.09
α-Elastin	15.89	0.36	0.07
β-Elastin	15.60	0.42	0.12
β-Elastin	15.60	0.40	0.12

Starch-gel electrophoresis. This was carried out according to Smithies [9] with some modifications necessary for separation of the soluble elastins. Starch was hydrolysed for only 37 min. and gel of only 10% concentration was prepared using 0.05 M-ammonium formate buffer of pH 3.0. The gel, 6 ml., was deposited on a glass plate (7.5×2.5 cm.) and left overnight in the cold. Electrophoresis was carried out in an ice-cooled chamber in 0.05 M-ammonium formate buffer of pH 3.0 for 45 min. at 220 V, the protein being applied as a 2% solution. The electrophoretograms were stained with 0.2% nigrosine solution in 0.05 N-HCl, then washed with water.

Analytical methods. Nitrogen was determined by the micro-Kjeldahl procedure and the amount of protein was calculated using a factor 6.37. Hexosamines were estimated by the method of Elson-Morgan in the modification of Blix [2]. Uronic acids were determined according to Dische [3]. Viscosity measurements at different pH values were carried out in Ostwald's type viscometer at a temperature of 25° ±0.03°, using as solvents 0.1 M-ammonium formate buffers in the pH range from 3.3 to 5.1, and 0.1 M-tris-HCl buffers in the pH range from 5.7 to 8.4. Limiting viscosity numbers were calculated from a plot of specific viscosity against protein concentration in g./ml., kinematic corrections being neglected.

Reagents. The reagents used were Polish commercial products of analytical grade purity. Cetylpyridinium chloride (CPC) was kindly supplied by Grodziskie Zakłady Farmaceutyczne through the courtesy of Mgr. L. Krupiński. The prepara-

tion corresponded to the requirements of U.S. Pharm. XVI, 1960. Tannic acid was a Mallincrodt (New York, U.S.A.) and nigrosine a B.D.H. (London, England) product.

RESULTS

The effect of CPC on the precipitation of α- and β-elastin with tannic acid. Tannic acid forms precipitates with α- as well as with β-elastin in aqueous solution. In Fig. 1 the relationship between the pH of the medium and the amount of tannic acid necessary for complete protein precipitation is presented.

As demonstrated by Mejbaum-Katzenellenbogen [5], proteins are quantitatively precipitated in very acid solution in the presence of tannic acid. Therefore it was assumed that precipitation of protein was complete when on addition of HCl to

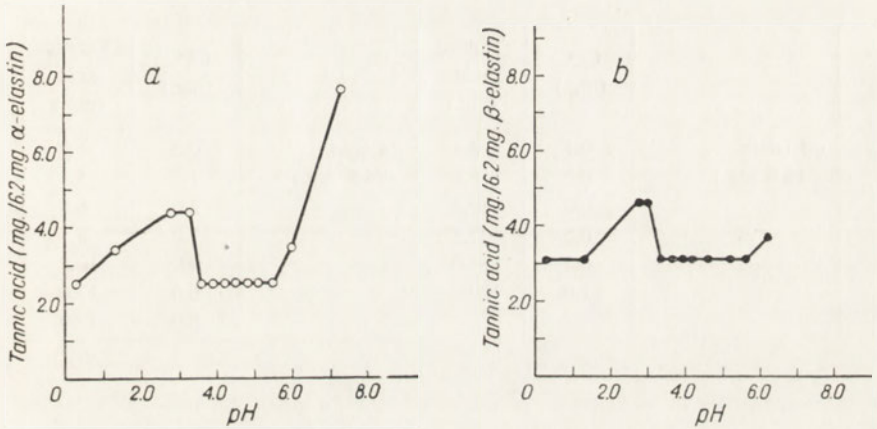

Fig. 1. Effect of pH on the amount of tannic acid necessary for complete precipitation of (*a*), α--elastin and (*b*), β-elastin. To 2 ml. of an elastin solution containing 6.2 mg. of protein, 0.31% solution of tannic acid was added until complete precipitation of protein occurred. The reaction was carried out for pH 0.3 in 1 N-HCl, for pH 1.3 in 0.1 N-HCl, for pH 2.8 - 3.3 in 0.1 M-ammonium formate buffer, and for pH 3.6 - 8.0 in 0.1 M-ammonium acetate buffer.

1 N concentration in the presence of an excess of tannic acid no more precipitate was formed. Analysis of the supernatant for nitrogen content also indicated that the precipitation was complete. It can be seen in Fig. 1 that at pH < 1 and in the pH region 3.6 - 5.5 the amount of tannic acid sufficient for precipitation of α- and β-elastin was the lowest.

Cetylpyridinium chloride (CPC) inhibited the formation of protein - tannic acid complexes. These experiments were carried out at pH 4 - 5 which is the optimum pH range for both proteins to form insoluble complexes with tannic acid. To a series of glass tubes containing 6.8 mg. of protein, increasing amounts of CPC were added. Then tannic acid was added dropwise until the first precipitate appeared. Quanti-

tative relationship between the amount of CPC and its ability to prevent the precipitation is presented in Table 2 and Fig. 2. These data show that the protective action of CPC against precipitation is more pronounced for α-elastin than for β-elastin (unhatched areas in Fig. 2). It should be pointed out that these results were obtained in aqueous solution of very low ionic strength (below I 0.001). In the presence of greater ion concentration the protective properties of CPC were diminished.

Table 2

Relationship between the amount of CPC present in the solution and the amount of tannic acid needed for appearance of the precipitate of α- or β-elastin

To 2 ml. of α- or β-elastin solution containing 6.8 mg. of protein, different volumes of 1% CPC solution were added. Then tannic acid solution was added dropwise until a precipitate appeared. The reactions were carried out in aqueous solution at pH 4-5.

	CPC (mg.)	Tannic acid (mg.)		CPC (mg.)	Tannic acid (mg.)
α-Elastin (6.8 mg.)	0.1	3.4	β-Elastin (6.8 mg.)	0.5	1.7
	0.2	6.8		1.0	5.1
	0.3	10.2		2.0	6.8
	0.5	27.2		3.0	8.5
	0.7	60.0		4.0	10.2
	1.0	100.0		6.0	17.0
				8.0	17.0

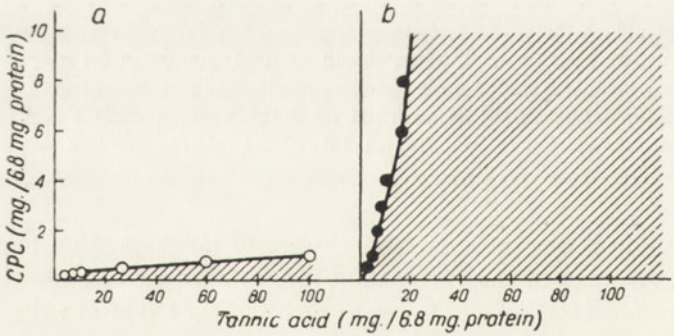

Fig. 2. Effect of CPC on the precipitation by tannic acid of (*a*), α-elastin and (*b*), β-elastin. Hatched areas represent the region of protein precipitation. Data from Table 2.

Effect of pH *on staining with nigrosine solution.* One drop of α- or β-elastin aqueous solutions of different concentration was placed on filter paper. After drying, the paper was immersed in 0.2% nigrosine solution of pH values ranging from

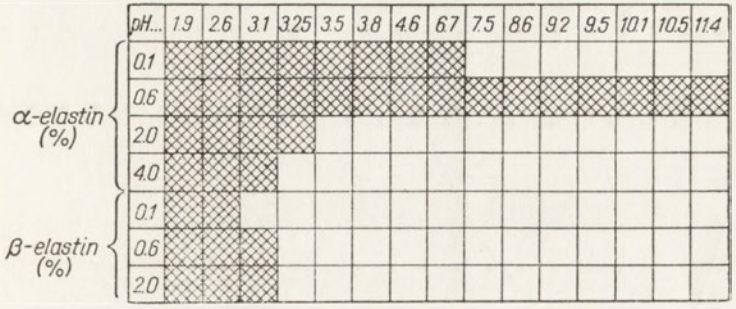

Fig. 3. Stainability of α- and β-elastin by nigrosine in relation to pH of the dye solution. Hatched areas represent the region of stainability.

1.9 to 11.4. The results presented in Fig. 3 show that β-elastin in concentration of 0.1 - 2.0% adsorbed the dye only at acidic pH values. On the other hand, α-elastin in 0.1% concentration was stained over pH values 1.6 - 6.7 and in 0.6% concentration even up to pH 11.4 but in greater concentration, similarly as β-elastin. A mixture of the two soluble proteins was stained similarly as α-elastin alone.

Starch-gel electrophoresis. No separation of α- and β-elastin could be obtained on paper or agar-gel electrophoresis over a broad range of pH and ionic strength

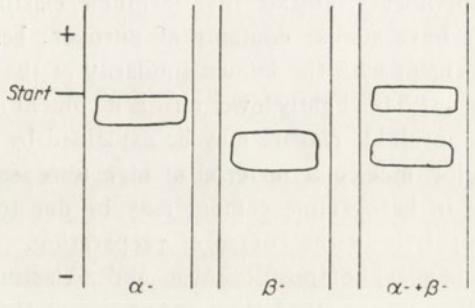

Fig. 4. Starch-gel electrophoretic patterns of α-elastin, β-elastin and their mixure.

values. On starch gel, however, at pH 3.0 satisfactory separation of the two proteins was obtained (Fig. 4). The electrophoretograms could be evaluated quantitatively using a reflection densitometer scanner.

Viscosity of soluble elastins. Limiting viscosity numbers were significantly higher for α-elastin than for β-elastin over the whole range of pH values tested (Fig. 5). The limiting viscosity number for α-elastin was pH-dependent whereas for β-elastin it was not dependent on the pH values. The lowest limiting viscosity number for α-elastin 9.15 was observed at pH 4.5; at this pH the corresponding value for β-elastin was 7.15.

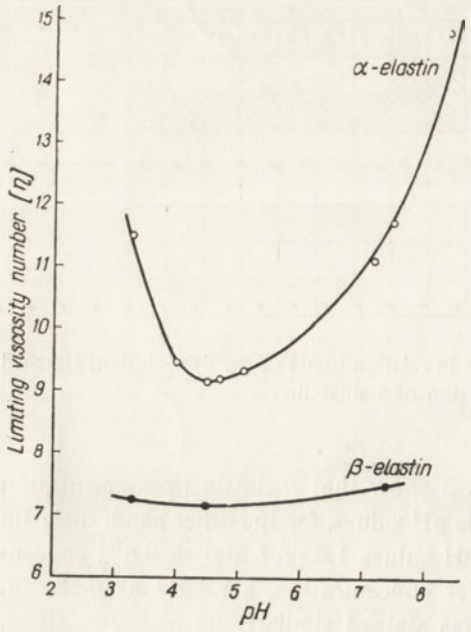

Fig. 5. The effect of pH on the limiting viscosity numbers of (○), α-elastin and (●), β-elastin.

DISCUSSION

The presented experiments indicate that insoluble elastin and its soluble decomposition products have similar contents of nitrogen, hexosamine and uronic acid, which is in agreement with the known similarity of the amino acid composition of α- and β elastin [8]. The slightly lower nitrogen content in the soluble proteins as compared with the insoluble elastin, may be explained by assuming that during the dissolution some low-molecular material of high nitrogen content is split off. The small differences in hexosamine content may be due to slight differences in the conditions of hydrolysis in the course of preparation.

The similarity of chemical composition of α- and β-elastin renders their distinction difficult. It seems, however, that other properties of these proteins described in this paper can be utilized for their differentiation. (1), β-Elastin was stained with nigrosine only at acid pH below 3.0 whereas α-elastin at 0.6% concentration stains even at an alkaline pH; however, at higher concentration the stainability of α-elastin was diminished due to the impregnation effect of this protein on the supporting paper. (2), At pH 3.0 on starch gel electrophoresis the two proteins separated quite satisfactorily but did not separate at higher pH values. (3), CPC inhibited precipitation by tannic acid, its protective effect being more pronounced for α-elastin than for β-elastin. The difference in the protective CPC concentration was large enough to enable differentiation and separation of the two soluble elastins. The amount of milligrams of CPC necessary to inhibit the precipitation of α-elastin was one tenth of that needed to inhibit the precipitation of β-elastin by the same amount of tannic acid.

Assuming the molecular weight of α-elastin to be 60 000 and of β-elastin 6000, the molecular relationship between protein, CPC (mol. wt. 340) and tannic acid (mol. wt. 1700) can be calculated. The figures presented in Table 3 show that at the same molar ratio of CPC to protein, the amount of tannic acid needed to precipitate α-elastin was about ten times greater than that needed to precipitate β-elastin.

Table 3

Molar relations between soluble elastin preparations, CPC, and tannic acid at the moment of appearance of the first precipitate

The figures are calculated on the basis of the experiments shown in Table 2.

$\frac{\text{Moles CPC}}{\text{mole protein}}$	$\frac{\text{Moles tannic acid}}{\text{mole } \alpha\text{-elastin}}$	$\frac{\text{Moles tannic acid}}{\text{mole } \beta\text{-elastin}}$	Mol. wt. of the protein fragment corresponding to one tannic acid molecule for:	
			α-elastin $\left(\frac{60\,000}{b}\right)$	β-elastin $\left(\frac{6\,000}{c}\right)$
a	*b*	*c*	*d*	*e*
2.6	18	2.7	3330	2220
5.2	36	3.5	1660	1700
8.0	54	4.4	1110	1360

The experiments described in this paper suggest that α- and β-elastin react with tannic acid like a polypeptide chain and the amount of tannic acid needed for precipitation is directly proportional to the length of the polypeptide chain. The two proteins, however, behave towards CPC as a distinct molecule species and the amount of detergent needed for the protection against precipitation is directly proportional to the number of molecules present in solution, independently of their molecular weight. The calculated molecular weight of the fragment of the elastin molecule which binds one tannic acid molecule indicates (Table 3) that the size of this fragment is similar for both α- and β-elastin, at a given concentration of protein, CPC and tannic acid. These values suggest a close relationship between the polypeptide chains of α- and β-elastin. In Fig. 6 is presented a suggested mode of interaction of CPC and tannic acid with protein.

Viscometric data furnish additional support to the explanation of the mechanism of interaction between the soluble elastins and CPC. The limiting viscosity numbers for α-elastin are pH-dependent, showing a clear cut minimum at pH 4.5. On the other hand, viscosity of β-elastin was practically not pH-dependent. The changes in the limiting viscosity numbers of α-elastin as a function of pH are in agreement with current views on the nature of coacervation [1, 7, 4]. The α-elastin molecules at pH about 4.5 may be easily coacervated by increasing the temperature. In the

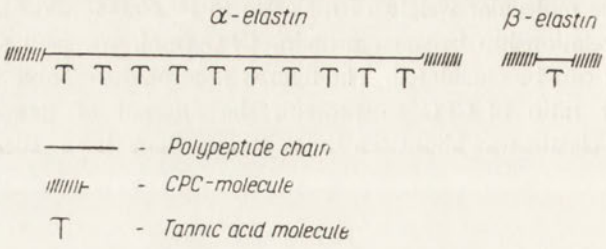

Fig. 6. Schematic diagram of the hypothetical interaction between α- and β-elastin, CPC and tanni acid.

presence of CPC a stabilization of the structure occurs and no coacervation can be observed. This may be explained by the role ascribed to hydrophobic bonds in protein - detergent complexes and in protein stability [6]. It may be assumed that the high content of non-polar amino acids in insoluble and soluble elastins enables the formation of numerous hydrophobic bonds with the non-polar residue of the detergent molecule. Thus the α-elastin molecule at pH about 4.5 may bind CPC molecules in the same manner as does the β-elastin molecule.

The authors are greatly indebted to Prof. Dr. E. Kowalski for his guidance in this work and to Prof. Dr. K. Zakrzewski for helpful discussions.

SUMMARY

α- and β-elastin were isolated from oxalic acid digest of insoluble elastin preparation from ox ligamentum nuchae, and it was found that they are precipitated by tannic acid over a wide pH range, the optimum conditions being below pH 1 and between pH 3.6 and 5.5. Cetylpyridinium chloride protects α-elastin against the precipitation in concentration amounting to about 1/10 of that needed for protection of β-elastin.

Nigrosine solution (0.2%) stains either protein below pH 3.1 but at higher pH values only α-elastin is stained. Different electrophoretical mobility in starch gel at pH 3.0 permits to separate the proteins.

Limiting viscosity numbers are pH-dependent only for α-elastin, with a minimum at pH 4.5.

REFERENCES

[1] Adair G. S., Davis H. F. & Partridge S. M. - *Nature* **167**, 605, 1951.
[2] Blix G. - *Acta Chem. Scand.* **2**, 467, 1948.
[3] Dische Z. - *J. Biol. Chem.* **167**, 189, 1947.
[4] Hall D. A. & Czerkawski J. W. - *Biochem. J.* **80**, 121, 1961.

[5] Mejbaum-Katzenellenbogen W. - *Acta Biochim. Polon.* **6**, 375, 1959.
[6] Nemethy C. & Sheraga H. A. - *J. Phys. Chem.* **66**, 1773, 1962.
[7] Partridge S. M., Davis H. F. & Adair G. S. - *Biochem. J.* **61**, 11, 1955.
[8] Partridge S. M. & Davis H. F. - *Biochem. J.* **61**, 21, 1955.
[9] Smithies O. - *Biochem. J.* **71**, 585, 1959.

NIEKTÓRE WŁASNOŚCI PRODUKTÓW DEGRADACJI ELASTYNY

Streszczenie

α- i β-elastynę otrzymano przez traktowanie kwasem szczawiowym nierozpuszczalnej elastyny uzyskanej z wołowego więzadła karkowego. Obydwa białka strącają się pod działaniem taniny w szerokim zakresie pH; najlepsze warunki strącania się są w pH $\leqslant 1$ oraz pomiędzy pH 3.6 a 5.5. Chlorek cetylopirydyniowy zapobiega wytrącaniu taniną α-elastyny w stężeniu dziesięciokrotnie mniejszym od stężenia zapobiegającego strącaniu β-elastyny.

Obydwa białka barwią się w 0.2% roztworze nigrozyny poniżej pH 3.1, lecz powyżej tego pH barwi się tylko α-elastyna. Różna ruchliwość elektroforetyczna w żelu skrobiowym przy pH 3.0 umożliwia rozdział tych białek.

Tylko dla α-elastyny graniczne liczby lepkości są zależne od pH, z minimum przy pH 4.5.

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MUTAGENICITY OF HYDROXYLAMINE: REACTION WITH ANALOGUES OF CYTOSINE, 5(6)-SUBSTITUTED CYTOSINES AND SOME 2-KETO-4--ETHOXYPYRIMIDINES

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Amongst the chemical mutagens currently under investigation hydroxylamine has been recently receiving increasing attention. In part this is due to its relatively low lethal effect as compared to its high degree of mutagenicity. Of equal importance have been the demonstrations that it reacts specifically with some pyrimidines, particularly uracil and cytosine [7, 8, 16, 22], whereas the naturally occurring purines in nucleic acids are unaffected by it.

It has been variously reported [7, 8, 16] that, of the naturally occurring pyrimidine bases, cytosine reacts readily, 5-hydroxymethylcytosine to a lesser extent, uracil undergoes ring opening; while 5-methylcytosine and thymine are only slightly susceptible, if at all. Alkylation of the N_1 nitrogen, or its involvement in a glycosidic linkage, do not fundamentally affect the nature of the reaction. However, the reaction is markedly pH dependent. In the neighbourhood of neutrality it is largely cytosine which reacts, whereas at pH 9.5 - 10 cytosine is relatively inert while uracil readily reacts.

Consequently the mutagenic effect of hydroxylamine (on DNA) at neutral pH appears to be due largely, and perhaps exclusively, to its reaction with the cytosine ring [7, 4, 21]. The starting point for the present investigation was the reported low activity of hydroxylamine against 5-hydroxymethylcytosine [7, 8, 16] as compared to its appreciable mutagenic effect on bacteriophage T_4 [7, 4], and the rather surprising claim that 5-methylcytosine is quite resistant to this reagent [7, 8, 16].

It has now been shown that 5-methylcytosine does in fact react with hydroxylamine in aqueous medium, and that previous failures to observe this reaction were due largely to the fact that the primary reaction product formed differs from that resulting from the treatment of cytosine with hydroxylamine [13]. These fiindings, which are presented in greater detail here, have been extended to embrace a more detailed study of the action of hydroxylamine on other selected pyrimidine derivatives with a view to a fuller elucidation of the nature of the reaction products with various cytosine analogues.

MATERIALS

Hydroxylamine hydrochloride was a commercial preparation which was recrystallized several times from ethanol to a constant melting point, 152 - 153°.

Cytosine was a Schuchardt (München, Germany) product; 5-methylcytosine and 5-hydroxymethylcytosine were gifts of Sigma (St. Louis, U.S.A.) and Cal-Biochem. (U.S.A.), respectively. We are indebted to Dr. C. B. Reese for a sample of 6-methylcytosine; this sample was contaminated with some other ultraviolet-absorbing impurity, which was readily removed by chromatography on paper.

4-Ethoxy-2-ketopyrimidine was prepared by the acid hydrolysis of 2,4-diethoxypyrimidine, as described by Hilbert & Jansen [10]. 1-Methyl-4-ethoxy-2-ketopyrimidine was obtained by treatment of 2,4-diethoxypyrimidine with methyl iodide according to Hilbert & Johnson [11]. Methylaminocytosine bismethylaminocytosine and 1-methyl-4-methylaminocytosine were all prepared by Dr. W. Szer by amination of the corresponding 4-ethoxy derivatives. Analogous procedures were employed for the preparation of 1,5-dimethyl-4-ethoxy-2-ketopyrimidine (m.p 109 - 111°) and 1,6-dimethyl-4-methoxy-2-ketopyrimidine (m.p. 140 - 141°), the latter of which was prepared by Mrs. K. Ziabicka. We are indebted to Dr. W. Szer for the sample of 5-methylcytidine.

METHODS

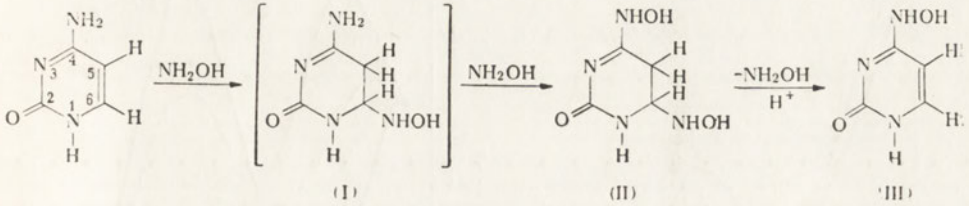
To follow the course of the reaction with hydroxylamine, the compound under study was dissolved at a concentration such that a 10 μl. sample, when added to a solution of 2.5 - 5.0 ml. buffered to a required pH, gave an extinction reading in a 10-mm. cuvette, at the appropriate wavelength in the ultraviolet, of about 0.5 - 0.8. The final concentration of pyrimidine in the reaction mixture varied therefore within the range 0.025 - 0.04 M. The hydroxylamine concentration was usually 2.5 M.

Hence to a 0.05 - 0.08 M aqueous solution of pyrimidine was added an equal volume of a 5 M aqueous solution of hydroxylamine hydrochloride which had been previously neutralized to the required pH. At given time intervals, samples were withdrawn for spectral observations and for paper chromatography. Spectral observations of the reaction mixtures are slightly complicated at wavelengths below 225 mμ, where neutral and alkaline solutions of hydroxylamine exhibit some absorption which changes with time. Spectral measurements of the course of the reactions were therefore reduced to the minimum in this wavelength region.

Under the foregoing conditions the rates of reaction with hydroxylamine were relatively rapid, e.g. with cytosine the reaction was virtually complete within 2 hr. at 37° and pH 6.5. For cytidine the corresponding time was about 3 - 4 hr. This is to be compared with that reported by Schuster [16] for a 100-fold lower concentration of cytidine in 1 M-hydroxylamine at 20° and pH 6.2, several days being required under these conditions.

RESULTS

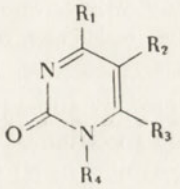
The nature of the reaction of hydroxylamine with cytosine and cytidine has been investigated by a number of observers [7, 8, 16, 22] and, at about neutral pH, has been formulated by Brown & Schell [2] as shown in Scheme 1. Compound II was obtained directly from the reaction mixture. At acid pH, II eliminates one



Scheme 1

mole hydroxylamine to give III; this acid-catalysed elimination proceeds slowly even in the neighbourhood of neutrality. Compound I itself was not isolated and was presumed to be an intermediate in the formation of II. The failure to detect I was believed to indicate that the rate of the exchange reaction I → II is equal to or exceeds that of, the addition reaction cytosine → I.

Additional light is thrown on the above postulated reaction sequence, cytosine → → [I] → II, by an examination, (a) of the reaction of III with hydroxylamine and, (b) of the reaction of 4-ethoxy-2-ketopyrimidine (IV, $R_1 = OC_2H_5$, $R_2 = R_3 = R_4 = H$) with hydroxylamine, as will be shown below.



(IV)

Scheme 2

The reaction between hydroxylamine and cytosine, as followed spectrally, is shown in Fig. 1, and is accompanied by a progressive decrease in the height of the characteristic absorption maximum at 267 mμ and the appearance of a new maximum at about 220 mμ (Note: for 5,6-dihydrocytosine at neutral pH the absorption maximum is at about 240 mμ, see [12]), testifying to saturation of the 5,6 double bond in the cytosine ring [12]. In agreement with Brown & Schell [2], acidification of this product led to the acid-catalysed elimination of hydroxylamine with reformation of the 5,6 double bond to give III. The spectrum of III at neutral and acid pH (neutral and cationic forms, respectively), and at one of the intermediate pH values used to determine its cationic p*K* by spectrophotometric titration (see Table 1), is shown in Fig. 2.

If, now, the isolated compound III is in turn treated with hydroxylamine, the latter undergoes addition to the 5,6 double bond of III. Saturation of the latter is shown by the disappearance of the characteristic absorption maximum at 270 mμ

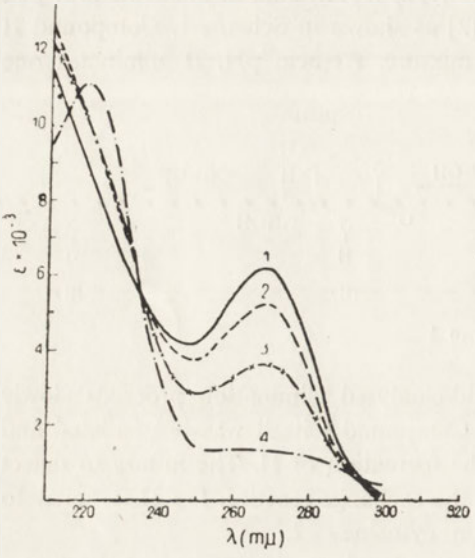


Fig. 1

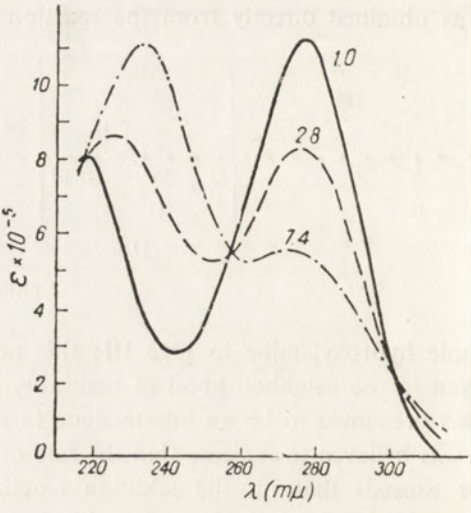


Fig. 2

Fig. 1. Course of reaction of cytosine with 2.5 M-hydroxylamine, pH 6.5, at 37°, as followed by changes in absorption spectrum measured at pH 7.4: 1, absorption spectrum at zero time, 2, 3 and 4, after resp. 15, 40 and 120 min. reaction.

Fig. 2. Absorption spectrum in aqueous medium of 4-hydroxylamino-2-ketopyrimidine at pH values indicated beside each curve.

(see Fig. 3). This is further confirmed by subsequent acidification of the solution to regenerate the cationic absorption spectrum of III. In particular, it will be noted that the rate of addition of hydroxylamine to III is slower than its rate of addition to cytosine. At pH 6.5, the addition reaction is in fact 5 to 10-fold slower than for

Table 1

Cationic and anionic apparent pK values for analogues of 4-hydroxylamino-2-keto-pyrimidine

Analogue	pK_a	pK_b
Parent compound	2.8	—
1-Methyl-	2.9*	10.4*
5-Methyl-	2.8	—
1,5-Dimethyl-	2.9**	11.1**
1,6-Dimethyl-	3.65	10.7
5-Hydroxymethyl-	2.2	—

* For the 1-(2-deoxy-β-D-ribofuranosyl)- analogue $pK_a = 2.26$, $pK_b = 10.5$ [6].
** For the 1-(β-D-ribofuranosyl)- analogue $pK_a = 2.3$, $pK_b = 11.1$ [6].

the corresponding addition to cytosine. Since the addition reaction to cytosine, followed spectrally, leads to formation of II, in which the amino group has also been replaced by the hydroxylamino group, it must be concluded that the first

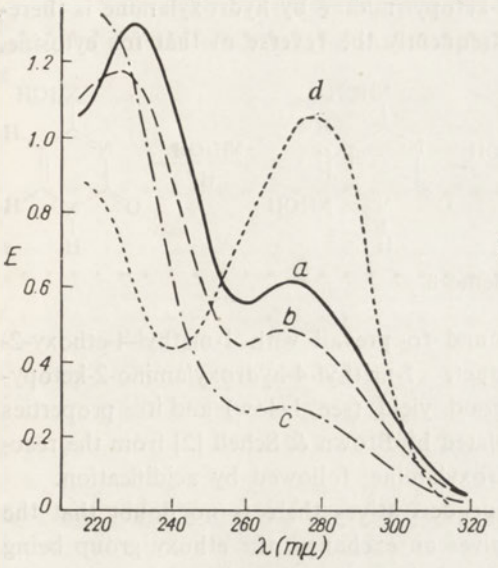


Fig. 3

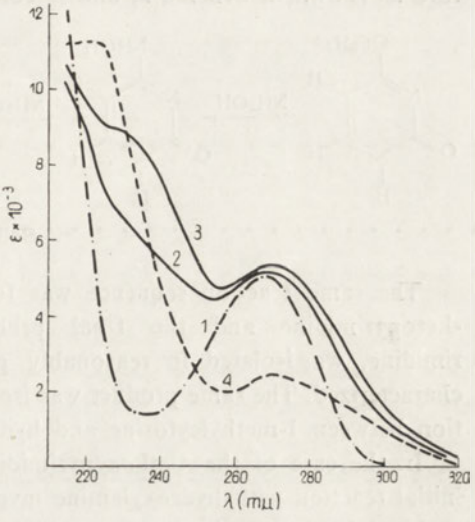


Fig. 4

Fig. 3. Course of reaction of 4-hydroxylamino-2-ketopyrimidine with 2.5 M-hydroxylamine at pH 6.5 and 37° due to addition of hydroxylamine at 5.6 bond, followed by elimination of hydroxylamine in acid. (*a*), Absorption spectrum at zero time, at pH 7.4; (*b*), following 3 hr. reaction, at pH 7.4; (*c*), following 18 hr. reaction, at pH 7.4; (*d*), following acidification of reaction mixture to 0.1 N-HCl and leaving for 20 hr. at room temp. From Fig. 2, this curve is seen to be the absorption spectrum of 4-hydroxylamino-2-ketopyrimidine at pH 1.

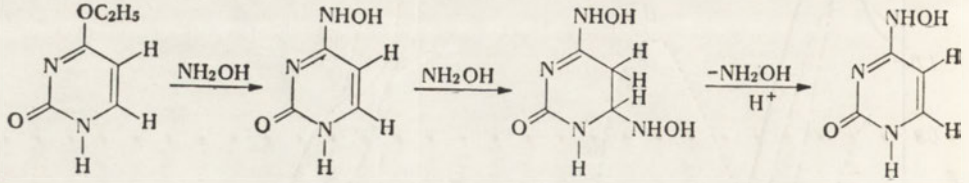
Fig. 4. Course of reaction of 4-ethoxy-2-ketopyrimidine with 2.5 M-hydroxylamine at pH 6.5 and 37°. Curves 1, 2, 3, 4 show absorption spectra (measured at pH 7.4) at the start of the reaction and following 1, 2 and 18 hr.

stage in the reaction of cytosine does not involve replacement of the amino group, and that the reaction scheme proposed by Brown & Schell [2], as shown above, is essentially correct.

The foregoing is further substantiated by an examination of the reaction with hydroxylamine of 4-ethoxy-2-ketopyrimidine. The course of this reaction was followed spectrally and is shown in Fig. 4, from which it will be readily noted that two successive reactions are involved. The first of these, which is complete in well under 2 hr., leads to the formation of a product with an absorption spectrum represented by curve 3, with a maximum at about 271 mμ. Prolongation of the reaction time provokes the gradual disappearance of this maximum (curve 4), but at a much lower rate.

Curve 3 is, in fact, the absorption spectrum of III (cf. Fig. 2, curve for pH 7.4) and compound III can indeed be isolated from the reaction mixture on a preparative scale in reasonably good yield (see below), its melting point, elementary analysis and other properties being identical with those obtained above from the reaction

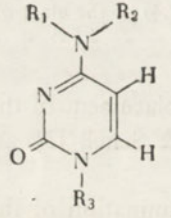
of cytosine with hydroxylamine. When the reaction time was prolonged to 24 - 48 hr., the product formed was shown to resemble II spectrally and, what is more convincing, was transformed back to III on treatment with acid. The reaction sequence involved in the transformation of 4-ethoxy-2-ketopyrimidine by hydroxylamine is therefore as shown in Scheme 3, and is consequently the reverse of that for cytosine.



Scheme 3

The same reaction sequence was found to prevail with 1-methyl-4-ethoxy-2--ketopyrimidine and the final product, 1-methyl-4-hydroxylamino-2-ketopyrimidine, was isolated in reasonably good yield (see below) and its properties characterized. The same product was isolated by Brown & Schell [2] from the reaction between 1-methylcytosine and hydroxylamine, followed by acidification.

In the case of the 4-ethoxypyrimidine derivatives there is no doubt that the initial reaction with hydroxylamine involves an exchange, the ethoxy group being replaced by hydroxylamine at position 4 of the pyrimidine ring. That the same exchange reaction occurs with the amino group in cytosine analogues was demonstrated in a more unequivocal manner by the use of alkylamino derivatives of cytosine corresponding to Va, Vb and Vc. With all three of these compounds (Scheme 4), the initial reaction products were the 4,6-bishydroxylamino-5,6-dihydro derivatives which, in acid, were converted to the 4-hydroxylamino derivatives.



(V)

(a) $R_1 = H$, $R_2 = CH_3$, $R_3 = H$

(b) $R_1 = CH_3$, $R_2 = CH_3$, $R_3 = H$

(c) $R_1 = H$, $R_2 = CH_3$, $R_3 = CH_3$

Scheme 4

The reaction products were not isolated on a preparative scale, but were identified by spectral and chromatographic procedures (Table 2). These results leave no doubt but that the amino group is completely removed to be replaced by the nucleophilic hydroxylamino-NHOH.

Quite different in character is the reaction course with cytosine analogues in which the 5- or 6-position is alkylated. As already pointed out in the Introduction, 5-methylcytosine was previously reported to be resistant to hydroxylamine [7,

Table 2

R_F *values on paper chromatography*

Ascending technique, with Whatman no. 1 paper. Solvents: *A*, water-saturated butanol; *B*, butanol saturated with 0.2 N-HCl; *C*, isopropanol, 36 ml.; conc. HCl, 8.2 ml.; and water to a total volume of 50 ml. Values in parentheses are for compounds deposited on the paper chromatograms from a hydroxylamine solution.

Compound	Solvent			
	A		*B*	*C*
Cytosine	0.22	(0.11)	—	0.44
4-Ethoxyuracil	0.71		0.67	0.77
N_4-Monomethylcytosine	0.41	(0.13)	0.16	0.48
N_4-Dimethylcytosine	0.41	(0.13)	0.08	0.45
4-Hydroxylamino-2-ketopyrimidine	0.40	(0.17)	0.16	0.49
5-Methylcytosine	0.28	(0.12)	0.11	0.62
5-Methyl-4-hydroxylamino-2-ketopyrimidine	0.54		0.11	0.67
5-Methylcytidine		(0.08)	0.04	0.51
5-Methyl-4-hydroxylamino-2-ketopyrimidine riboside		(0.42)	0.07	0.62
5-Hydroxymethylcytosine	0.12		0.05	0.42
5-Hydroxymethyl-4-hydroxylamino-2-ketopyrimidine	0.25		0.07	0.41
6-Methylcytosine		(0.15)	0.09	0.59
6-Methyl-4-hydroxylamino-2-ketopyrimidine		(0.6)	0.22	0.67
1-Methyl-4-ethoxyuracil	0.84		0.86	0.8
1-Methyl-N_4-monomethylcytosine	0.47		0.52	0.66
1-Methylcytosine				0.59
1-Methyl-4-hydroxylamino-2-ketopyrimidine	0.47		0.55	0.58
1,5-Dimethyl-4-ethoxyuracil	0.88		0.83	0.91
1,5-Dimethyl-4-hydroxylamino-2-ketopyrimidine	0.53		0.56	0.61
1,6-Dimethyl-4-methoxyuracil	0.73		0.80	0.74
1,6-Dimethyl-4-hydroxylamino-2-ketopyrimidine	0.2	(streak)	0.48	0.55

8, 16], this conclusion being based on the fact that the height of the principal absorption maximum at 275 mμ remains practically unaltered in the presence of hydroxylamine. However, as already shown elsewhere [13], 5-methylcytosine does react with hydroxylamine under the same conditions as cytosine. From Fig. 5, which exhibits the modifications in absorption spectrum resulting from this reaction, it is at once clear why previous efforts, based solely on observations at 275 mμ, were negative, since the extinction at this wavelength is practically unaffected. At no point during the course of this reaction is there any disappearance of the characteristic absorption spectrum as for cytosine, so that no addition of hydroxylamine to the 5,6 double bond could have occurred. Isolation of the reaction product (see below), the absorption spectrum of which at different pH values is shown in Fig. 6, showed that only the amino group had undergone replacement according to Scheme 5. This is in accord with the known fact that cytosine and its glycosides, on irradiation with ultraviolet light, undergo nucleophilic addition of water to the

5,6 double bond, with resultant saturation of the latter [18, 20, 19, 24, 5, 14]; whereas 5-substituted cytosines do not [24].

The rate of reaction of 5-methylcytosine with hydroxylamine is approximately 5-fold lower than that for cytosine. With the riboside of 5-methylcytosine, both

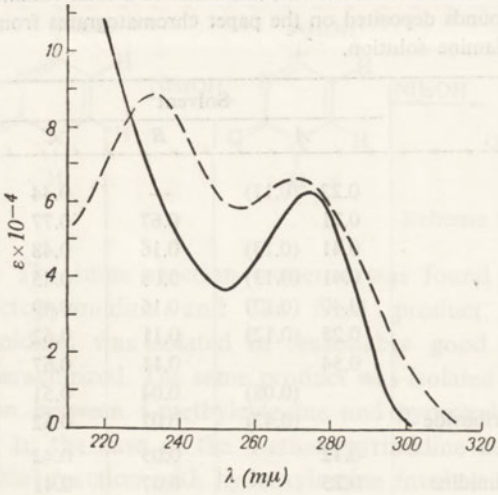


Fig. 5. Absorption spectrum at pH 7.4 of 5-methylcytosine: (———), prior to reaction with hydroxylamine; (— — — —), following reaction with hydroxylamine.

the nature of the reaction as well as the rate were found to be essentially the same as for 5-methylcytosine. The reaction in this case was followed by spectrophotometric methods and the product also checked chromatographically (see Table 2). The

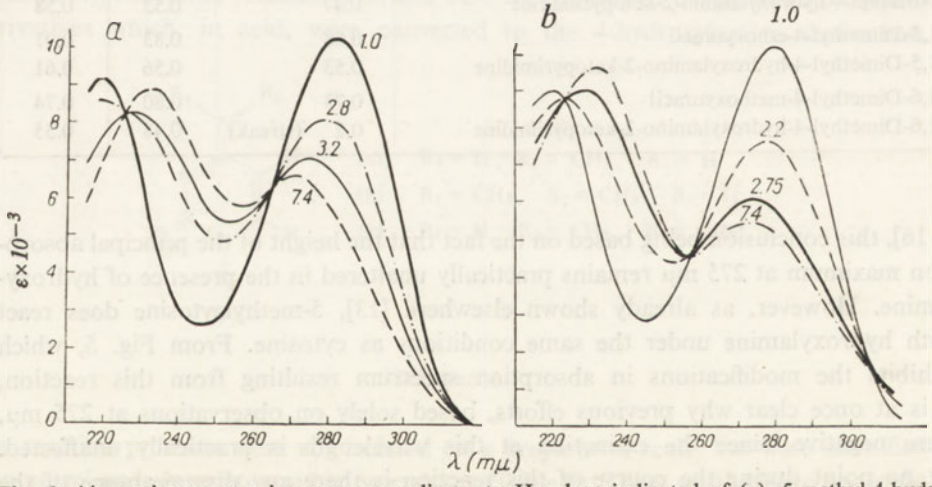
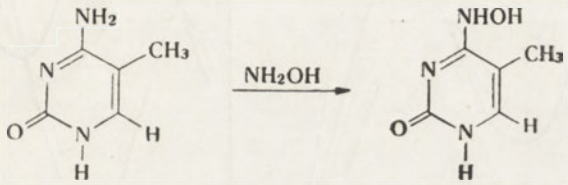


Fig. 6. Absorption spectra in aqueous medium at pH values indicated of (*a*), 5-methyl-4-hydroxylamino-2-ketopyrimidine; and (*b*), 5-hydroxymethyl-4-hydroxylamino-2-ketopyrimidine.

spectrum of the product was in full agreement with that to be expected from the spectrum of the 4-hydroxylamino analogue of 5-methylcytosine deoxyriboside reported by Fox *et al.* [6] and obtained by treatment with hydroxylamine of 4--thiothymidine.

Unlike 4-hydroxylamino-2-ketopyrimidine (III), 5-methyl-4-hydroxylamino-2--ketopyrimidine was unaffected by further treatment with hydroxylamine, in agreement with expectations. Furthermore, 6-methylcytosine was found to exhibit the same behaviour, its only reaction product with hydroxylamine being the 4-hydroxylamino analogue. In agreement with this, it was found that ultraviolet irradiation of 6-methylcytosine in aqueous medium did not lead to uptake of a water molecule.

It was found that 5-hydroxymethylcytosine, like 5-methylcytosine, undergoes only exchange of the amino group to give the 4-hydroxylamino derivative, the spectrum of which (Fig. 6b) is quite analogous to that for the 4-hydroxylamino derivative of 5-methylcytosine (Fig. 6a).



Scheme 5

1,5-Dimethyl-4-ethoxy-2-ketopyrimidine (IV, $R_1 = OC_2H_5$, $R_2 = R_4 = CH_3$, $R_3 = H$) and 1,6-dimethyl-4-methoxy-2-ketopyrimidine (IV, $R_1 = OC_2H_5$, $R_2 = H$, $R_3 = R_4 = CH_3$) both reacted with hydroxylamine to give, in each case, the corresponding 4-hydroxylamino derivative. Both of these were isolated in quantitative yield (see below) and their spectra at various pH values are shown in Fig. 7. Neither of them, on treatment with hydroxylamine, showed any evidence of addition of this reagent at the 5,6 bond.

Comparative measurements of the rate of exchange of the 4-alkoxy group by hydroxylamine showed that this was largely unaffected by alkyl substitution on C_5 or C_6. An examination of the reaction kinetics for transformation of the various derivatives would probably be of some value in the interpretation of the reaction mechanisms. In particular it would be of interest to examine the action of hydroxylamine on 4-thiouracil, 5-methyl-4-thiouracil and their glycosides in aqueous medium; in anhydrous medium at elevated temperatures these have been shown to react with hydroxylamine to give the corresponding 4-hydroxylamino derivatives [6].

Dissociation constants of 4-hydroxylamino derivatives. The cationic pK values of the various 4-hydroxylamino derivatives may be readily measured by spectrophotometric titration methods, as is clear from an examination of the spectra in the various figures. Values obtained are listed in Table 1. It should be noted, from an examination of the spectra of the various derivatives, that the fully protonated forms exhibit absorption spectra strikingly similar to those for the corresponding protonated cytosine derivatives [cf. 5], from which it may be reasonably inferred that they all protonate on the ring N_3 nitrogen, and not on the hydroxylamino group.

From Table 1 it will be noted that the cationic pK values for the 4-hydroxylamino derivatives are appreciably reduced as compared to those for the corresponding 4-amino (i.e. cytosine) analogues. Particularly striking is the low value for 5-hydroxy-

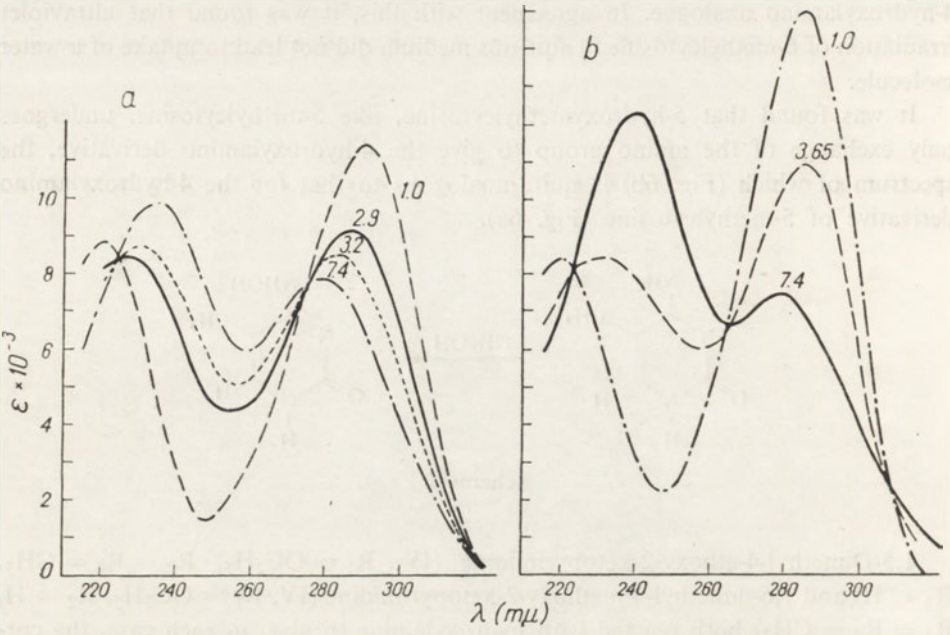


Fig. 7. Absorption spectra in aqueous medium at pH values indicated of (*a*), 1,5-dimethyl-4-hydroxylamino-2-ketopyrimidine; and (*b*), 1,6-dimethyl-4-hydroxylamino-2-ketopyrimidine.

methyl-4-hydroxylamino-2-ketopyrimidine (2.2) as compared to 5-methyl-4-hydroxylamino-2-ketopyrimidine (2.8); and the high value of the 1,6-dimethyl analogue (3.65) in comparison to that for the 1,5-dimethyl derivative (2.9).

In alkaline medium all of the above compounds dissociate by removal of a proton from the hydroxylamino group [6]. Because of the accompanying lability of the anions at high pH values, the dissociation constants were measured only for several of them and are also exhibited in Table 1. The corresponding spectra from which these dissociation constants were calculated are shown in Fig. 8 for two compounds.

It should be noted that the anionic pK values listed in Table 1 are for those compounds alkylated on the ring N_1 nitrogen. These are relatively stable at pH values up to about 13 at room temperature, as are the corresponding glycosides [6]. Even in 0.1 N-NaOH at room temperature, signs of instability are evident only after several hours.

When the ring N_1 nitrogen is unsubstituted, alkaline decomposition is very rapid. For example, compound II in 0.1 N-NaOH at 25° undergoes probably ring opening, with the concomitant disappearance of its absorption maximum at 235 mμ, the $t_{1/2}$ for this reaction being 70 min. The rate of decomposition of 4-hydroxylamino-2-ketopyrimidine (III) is also relatively rapid; in this instance there is at

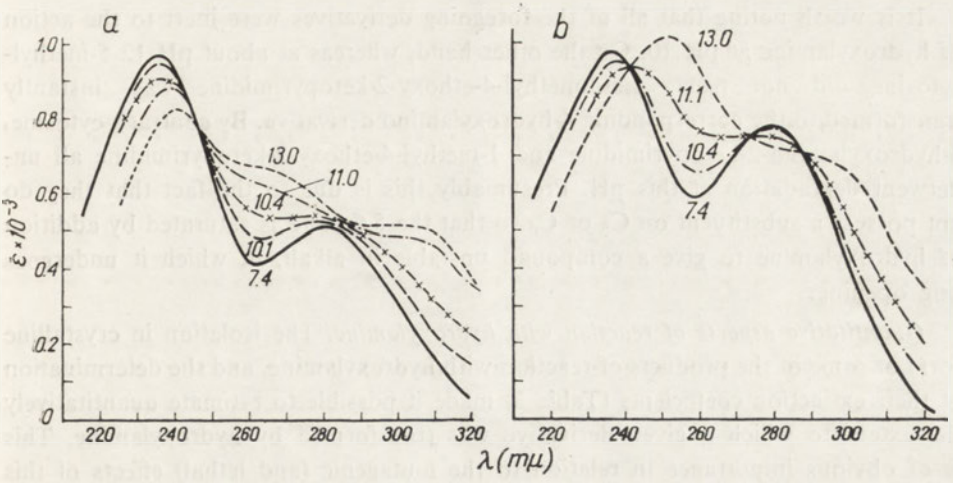


Fig. 8. Absorption spectra in aqueous medium at alkaline pH values indicated beside each curve of (*a*), 1-methyl-4-hydroxylamino-2-ketopyrimidine; and (*b*), 1,5-dimethyl-4-hydroxylamino-2--ketopyrimidine.

least partial hydrolytic removal of the hydroxylamino group, with resultant formation of uracil. No attempts were made to study this reaction in more detail or to determine what the other products were.

Influence of pH *on rate of reaction with hydroxylamine.* It was observed by Bautz-Freese & Freese [1] that the mutagenic effect of hydroxylamine on the transforming DNA of *Bacillus subtilis* was significantly enhanced at pH 4.2 as compared to pH 6.2, from which the authors concluded that this was due to a decrease in the p*K* of the cytosine residued in DNA as compared to free cytosine. If this were indeed so, one would expect to find the optimum reaction rate for a given compound at a pH corresponding roughly to its p*K*. This was tested for several compounds with varying p*K* values, including cytosine (p*K* 4.4), 5-methylcytosine (p*K* 4.6), 4-hydroxylamino-2-ketopyrimidine (p*K* 2.8); and 1-methyl-4-ethoxy-2-ketopyrimidine and 1,5-dimethyl-4-ethoxy-2-ketopyrimidine. Relative reaction rates with hydroxylamine were examined at the following pH values: 5.2, 6.5, 7.5 and 10 and 12.

It was found that for cytosine, 5-methylcytosine and 1,5-dimethyl-4-ethoxy--2-ketopyrimidine, the optimum reaction rate was at pH 6.5; whereas for 4-hydroxylamino-2-ketopyrimidine and 1-methyl-4-ethoxy-2-ketopyrimidine the optimum rate was at more alkaline pH values. It seems more likely that the shift of the optimum reaction rate for some compounds is more apparent than real, inasmuch as the concentration of free hydroxylamine increases with an increase in pH.

It follows that the increased mutagenicity of hydroxylamine at acid pH is not due to an increase in reactivity of cytosine residues. While no fully satisfactory interpretation for this effect is forthcoming, it is conceivable that it is due to an increase in the rate of formation of III, which is an acid-catalysed reaction.

It is worth noting that all of the foregoing derivatives were inert to the action of hydroxylamine at pH 10. On the other hand, whereas at about pH 12 5-methylcytosine did not react, 1,5-dimethyl-4-ethoxy-2-ketopyrimidine was instantly transformed to the corresponding 4-hydroxylamino derivative. By contrast, cytosine, 4-hydroxylamino-2-ketopyrimidine and 1-methyl-4-ethoxy-2-ketopyrimidine all underwent degradation at this pH. Presumably this is due to the fact that they do not possess a substituent on C_5 or C_6 so that the 5,6 bond is saturated by addition of hydroxylamine to give a compound unstable in alkali, in which it undergoes ring opening.

Quantitative aspects of reaction with hydroxylamine. The isolation in crystalline form of some of the products of reaction with hydroxylamine, and the determination of their extinction coefficients (Table 3) made it possible to estimate quantitatively the extent to which a given derivative was transformed by hydroxylamine. This is of obvious importance in relation to the mutagenic (and lethal) effects of this reagent.

Table 3

Spectral constants for some 4-hydroxylamino pyrimidine derivatives

Compound	pH	λ_{max} (mμ)	$\varepsilon_{max} \times 10^{-3}$
4,6-Bishydroxylamino-2-keto-5,6-dihydro-pyrimidine	1-7.4	220	12.0
4-Hydroxylamino-2-ketopyrimidine	1.0	218, 275	8.0, 11.25
	7.4	232, 273	11.0, 5.52
4-Hydroxylamino-5-methyl-2-ketopyrimidine	1.0	218, 280	9.12, 10.37
	7.4	232, 270	8.87, 6.62
1,5-Dimethyl-4-hydroxylamino-2-ketopyrimidine	1.0	220, 290	8.85, 11.43
	7.4	235, 280	9.87, 7.83
1,6-Dimethyl-4-hydroxylamino-2-ketopyrimidine	1.0	220, 285	8.39, 14.99
	7.4	235, 280	11.85, 7.49
4-Hydroxylamino-5-hydroxymethyl-2--ketopyrimidine	1.0	220, 280	9.0, 10.02
	7.4	230, 273	9.62, 5.43

The formation of 4-hydroxylamino derivatives was used as the basis for estimating the extent to which a given compound was transformed. In those instances where the primary product contained a saturated 5,6 bond, due to addition of hydroxylamine, this was transformed to the 4-hydroxylamino derivative either by heating at 90 - 95° for 15 min., or leaving at room temperature for 20 hr., in 0.1 N-HCl. It was observed that heating under the foregoing conditions resulted in the appearance of 1 - 5% of uracil, but this did not substantially affect the results outlined below.

It was in fact found that formation of 4-hydroxylamino products from cytosine and cytidine was not quantitative, but was in each case about 28% less than that expected theoretically. With the aid of a crystalline sample of II, it was found that

more than one-half of this loss occurred during the elimination of a molecule of hydroxylamine in acid to give III.

By contrast, in the case of 5-methylcytosine, where there is no addition of hydroxylamine to the 5,6 bond, formation of the 4-hydroxylamino derivative was fully quantitative.

Preparative isolation of reaction products

For isolation of reaction products on a preparative scale, it was found much more convenient in some instances to conduct the reaction in methanol [cf. 6], notwithstanding the longer reaction times necessary. Methanol proved to be a more suitable medium for crystallization of the products of reaction; furthermore, the large quantity of salt resulting from neutralization of hydroxylamine hydrochloride is absent under these conditions, thus further simplifying isolation of the products. Preparative procedures in aqueous medium were therefore employed for those compounds the reaction products of which were only slightly soluble in water and which consequently readily crystallized out.

Reaction product of 5-methylcytosine (4-hydroxylamino-2-keto-5-methylpyrimidine); 150 mg. of the HCl salt of 5-methylcytosine was dissolved in 24 ml. of 2.5 M aqueous hydroxylamine. After about 18 hr., by which time the reaction was complete, crystals began to deposit. The mixture was then transferred to the refrigerator for several hours, following which the crystals were filtered off, washed with water and then with methanol, and dried. Yield 91 mg. (70%), m.p. 261 - 262°. Elementary analysis: found: C, 42.5; H, 5.6; N, 29.3; calculated for $C_5H_7N_3O_2$: C, 42.6; H, 5.0; N, 29.8%. No attempt was made to increase the yield by repeated crystallization from the mother liquor.

Reaction product of 1,6-dimethyl-2-keto-4-methoxypyrimidine (1,6-dimethyl-4--hydroxylamino-2-ketopyrimidine); 100 mg. of 1,6-dimethyl-2-keto-4-methoxypyrimidine was dissolved in 10 ml. of 2.5 M aqueous hydroxylamine. The reaction under these conditions went to completion in about 2 hr., but crystallization began before this time. The reaction mixture was stored at 0° and the resulting crystals filtered off, washed and dried to give 76 mg. (yield 75%), m.p. 260 - 261°. Analysis: found, C, 46.22; H, 5.88; N, 26.34; calculated for $C_6H_9N_3O_2$: C, 46.1; H, 5.8; N, 26.9%.

Preparation of methanolic solutions of hydroxylamine: 17.5 g. of the HCl salt of hydroxylamine was dissolved in 150 ml. hot methanol. The solution was cooled in an ice bath and saturated sodium methoxide in methanol added dropwise with constant stirring until the pH was approximately 6.5. The precipitated salt was removed by filtration under vacuum and the filtrate, approximately 1.6 M-hydroxylamine, was stored in the deep freeze at -15°.

Reaction product of 1,5-dimethyl-4-ethoxy-2-ketopyrimidine (1,5-dimethyl-4-hydroxylamino-2-ketopyrimidine); 300 mg. of 1,5-dimethyl-4-ethoxy-2-ketopyrimidine was dissolved in 20 ml. of 1.6 M-hydroxylamine in methanol. Following

about 18 hr. the reaction was 80% complete, and went to completion in between 2 to 3 days. The solution was then evaporated on a water bath to about half its volume; the resulting precipitate, which exhibited no absorption in the ultraviolet, was filtered off. The filtrate was evaporated to dryness and dissolved in a minimum amount of hot methanol. On cooling, crystallization proceeded to give 94 mg. of washed and dried material. To the filtrate was added 1 N-HCl to about pH 1 on reduction of the volume, crystallization again took place to give 54 mg. of the HCl salt of 1,5-dimethyl-4-hydroxylamino-2-ketopyrimidine. Combined yield about 50%; m.p. of the free product, 214 - 215°; m.p. of the HCl salt, 208 - 210°. The free product gave on analysis, C, 46.25; H, 5.93; N, 26.41; calculated for $C_6H_9N_3O_2$: C, 46.1; H, 5.8; N, 26.9%.

Reaction product from 4-ethoxy-2-ketopyrimidine (4-hydroxylamino-2-ketopyrimidine); 300 mg. 4-ethoxy-2-ketopyrimidine was dissolved in 22 ml. of a 1.6 M-solution of hydroxylamine in methanol and the reaction allowed to go to completion (about 2, 3 days). Crystallization was then completed at 0° to give 164 mg. of washed and dried material. The mother liquor contains a mixture of 4-hydroxylamino-2--ketopyrimidine and 4,6-bishydroxylamino-2-keto-5,6-dihydropyrimidine. The latter was transformed to the former by adjusting the solution to pH 1 with HCl and heating for 15 min. at 100°. The solution was then brought to dryness and dissolved in the minimum amount of hot methanol; cooling resulted in a small crop of crystals, 21 mg. of the HCl salt of 4-hydroxylamino-2-ketopyrimidine. Total yield, about 70%; m.p. of free compound 243 - 244°. The HCl salt melted sharply, m.p. 214.5 - - 215.5°. For the HCl salt analysis gave: C, 28.7; H, 4.00; N, 26.0; calculated for $C_4H_5N_2O_2 \cdot HCl$: C, 29.2; H, 3.69; N, 25.6%.

Reaction product from cytosine (4-hydroxylamino-2-ketopyrimidine); 250 mg. cytosine was dissolved with heating in 35 ml. of a methanolic solution of 1.6 M-hydroxylamine. A small quantity of crystals appeared on the following day. The reaction was allowed to proceed for an additional 48 hr., following which the crop of crystals was filtered off, washed with water and methanol and dried to give 55 mg. 4,6-bishydroxylamino-2-keto-5,6-dihydropyrimidine. The mother liquor, which contained a mixture of the same compound plus 4-hydroxylamino-2-ketopyrimidine, was acidified and heated 15 min. at 90 - 95°, then cooled and neutralized. It is important to stir the solution during this step because of the alkaline lability of the product. Crystallization commences during neutralization; yield of crystals, 120 mg. Total yield 55%, m.p. of free compound 243 - 244°, as for the same compound obtained from 4-ethoxy-2-ketopyrimidine (see preceding paragraph).

The melting point of the bishydroxylamino derivative was found to vary with the rate of heating. When heated rapidly, it melted at 163 - 165°; on slow heating, it melted at 249 - 250°. These differences are probably related to the observation by Brown & Schell [3] that II is transformed to III on heating *in vacuo*. Undoubtedly the same transformation takes place on slow heating in the melting point capillary.

Reaction product from 1-methyl-4-ethoxy-2-ketopyrimidine (1-methyl-4-hydroxylamino-2-ketopyrimidine); 86 mg. of 1-methyl-4-ethoxy-2-ketopyrimidine was

dissolved in 4 ml. methanolic hydroxylamine. Crystallization set in during the course of the reaction. When this had gone almost to completion (about 3 days), the mixture was brought to 0°, following which the crystal crop was collected by filtration and washed to give 40 mg. of material. Following partial concentration of the mother liquor, an additional 8 mg. was harvested. Total yield 60%, m.p. 224 - 225°.

Reaction product from 5-hydroxymethylcytosine (4-hydroxylamino-5-hydroxymethyl-2-ketopyrimidine). The isolation of this product on a preparative scale proved somewhat difficult because of the small quantity of starting material at our disposal and the extremely high solubility of the product in water and alcohol. From 30 mg. of 5-hydroxymethylcytosine in 15 ml. of methanolic hydroxylamine, the reaction product was crystallized by concentration to give 7 mg. of a crystalline product which darkened at 145° without melting sharply. Any contamination present must, however, have been small since the extinction coefficient of the reaction product (Fig. 6b) agreed within 1% with that estimated from the conversion of 5-hydroxymethylcytosine to the same product, measured directly in a spectrophotometer cuvette.

DISCUSSION

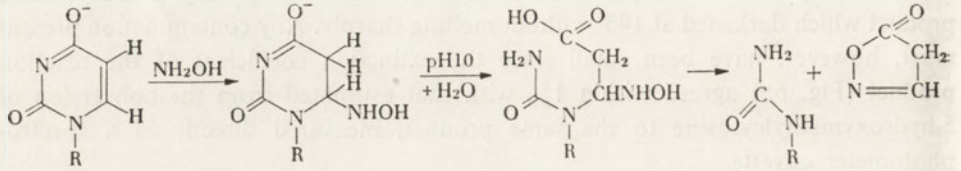
It seems reasonably well established from the above results that, in the case of cytosine and its glycosides, the initial attack of hydroxylamine involves addition of this latter to the 5,6 bond of the pyrimidine ring, and this in turn is followed by exchange of the amino group for an additional hydroxylamine molecule. In the case of 5-substituted cytosine and their glycosides, the only reaction that occurs (in aqueous medium under the same conditions) is exchange of the amino group for hydroxylamine. The findings with the alkylamino cytosine analogues establish beyond doubt that the exchange involves removal of the amino group and not exchange of an amino H for OH.

Following completion of the above work, Brown & Schell [3] reported that 5-methylcytosine and 5-hydroxymethylcytosine give primary products fully analogous to those of cytosine when subjected to the action of anhydrous hydroxylamine at elevated temperatures. However, what is primarily of interest from a biological point of view is the action of hydroxylamine under conditions where the latter manifests mutagenic activity, i.e. in aqueous medium at pH values corresponding to biological activity.

It remains, of course, to determine whether the reactions undergone in aqueous medium by the cytosine and 5-methylcytosine glycosides are reproduced when these components are incorporated in polynucleotide chains. Undoubtedly the most suitable model systems for such studies would be the synthetic ribo and deoxyribo homopolymers such as poly-C, poly-5MeC, as well as their twin-stranded complexes with the complementary polynucleotides poly-I and poly-G.

Some comment is called for with regard to the oft reported specificity of hydroxylamine for cytosine and its glycosides at neutral pH. Uracil and uridine have been

variously observed to react with hydroxylamine at alkaline pH, i.e. when they are in the form of monoanions. However, from the observations of Verwoerd *et al.* [23], it appears that uracil also reacts, albeit slowly, at neutral and even slightly acid pH. This is further substantiated by the reported action of hydroxylamine at neutral pH on the twin-stranded complex poly (A+U) [22]. Verwoerd *et al.* [23] and Schuster [16] found that the principal reaction products of uracil with hydroxylamine included iso-oxazolone. If we bear in mind the relative resistance of thymine to hydroxylamine, as compared to uracil, it seems reasonable to expect that the action of hydroxylamine on the latter involves nucleophilic attack at the 5,6 bond. At alkaline pH this would be followed by ring opening at the 3,4 bond, as in 5,6-dihydro-2,4-diketopyrimidines (see Scheme 6). It does not, of course, follow that



Scheme 6

the same reaction sequence prevails at neutral pH. We have, in fact, found in agreement with Verwoerd *et al.* [23] that uracil does react with hydroxylamine at pH 6.5 - 7.5 but have made no attempt to identify the reaction products. If the reaction is accompanied by ring opening and occurs also with uracil residues in, for example, plant viruses, it could in part explain the lethal effect of hydroxylamine, as observed in the case of infectious RNA from TMV [17]. It would obviously be desirable to examine the reaction products of uracil with hydroxylamine at neutral pH.

Thymine is relatively resistant to hydroxylamine in aqueous medium. This is in agreement with the observed resistance of 5-methylcytosine to attack at the 5,6 bond as compared to cytosine, and with the known resistance of thymine to photohydrate at the 5,6 bond as compared to uracil, which readily undergoes such photohydration [20]. It seems reasonable, consequently, to assume that the mutagenic action of hydroxylamine on DNA is due to its specific reaction with cytosine (or 5-substituted cytosine) residues. It would be of considerable interest, in this connection, to examine the action of hydroxylamine on a DNA such as that from the PBS2 bacteriophage, which is known to contain uracil in place of thymine; this should be accompanied by an enhanced lethal effect as compared to that for thymine-containing DNA.

Coming back now to the action of hydroxylamine on cytosine, the products include compounds such as II or III; and ring degradation should be also considered. It is clear that only the first two could result in mutagenic effects. The known mutagenic effects of hydroxylamine on T-even bacteriophages [7, 4], which contain only 5-substituted cytosines, suggest III as a likely candidate. Several observations argue in favour of III as the chemical species responsible for mutagenesis in normal

DNA. For example, mutations in normal transforming DNA are about 1000-fold less frequent than in T_4 bacteriophages [9]. Furthermore, transforming DNA subjected to the action of hydroxylamine exhibits a larger number of mutants at pH 4.5 than 6.5 [1], i.e. under acid conditions favouring the transformation of II to III.

However, the foregoing are by no means conclusive arguments. It has been shown that photochemically induced hydration of the 5,6 bond of cytosine residues in poly-C is sufficient to induce base-pair transitions in model *in vitro* replicating systems [15]. Addition of hydroxylamine to the 5,6 bond of cytosine might reasonably be expected to produce an analogous effect. Preliminary trials with hydroxylamine-treated poly-C (Grossman, personal communication, 1965) have demonstrated the induction of base-pair transitions similar to those produced by photohydration; however, it will have to be demonstrated that the exchange reaction of the amino group is not involved. In the case of poly-C, which exhibits only a moderate degree of secondary structure, the exchange reaction can probably take place. However, in a native DNA molecule, where the cytosine amino group is involved in hydrogen bonding, this reaction could conceivably be inhibited, or even absent. The results of Schuster [16] on the DNA of a T-even bacteriophage suggest that the exchange reaction does occur; but further experiments in this direction are required. It should be pointed out that the replacement of the cytosine amino group by hydroxylamine need not impair the ability of cytosine to form hydrogen bond with guanine; the presence of a hydroxyl group would affect only the strength of the hydrogen bond.

At least one simple experiment suggests itself for a test of the relative importance of II and III in mutagenic effects, viz. treatment of transforming DNA with hydroxylamine at pH 6.5, followed by acidification which favours the transformation of II to III. A comparison of the nature and number of mutants prior to and following acidification should indicate the relative importance of II and III.

Another reasonable possibility is to prepare 4-hydroxylamino-CDP. If this proved to be a substrate for polynucleotide phosphorylase, the resulting polymer could be tested in the DNA-controlled RNA polymerase system to determine whether the exchange reaction plays any role in mutagenesis.

SUMMARY

A study has been made of the action of hydroxylamine, in aqueous neutral medium, on cytosine, 5-substituted cytosines, their glycosides, on various alkyl--substituted cytosines, and on model 2-keto-4-ethoxy pyrimidines.

The action of hydroxylamine on cytosine involves addition of this reagent to the 5,6 bond; this is rapidly followed by removal of the amino group and its replacement by a second hydroxylamine molecule. The former may be eliminated in acid.

In 5-substituted cytosines and their glycosides, addition to the 5,6 bond does not occur, but only exchange of the amino group for hydroxylamine. This reaction is fully quantitative. The same behaviour is exhibited by 6-substituted cytosines.

The nature of the reactions involved has been examined by use of a variety of 4-ethoxy-2-ketopyrimidine analogues, which react with hydroxylamine.

The properties of the cytosine and 5-methylcytosine reaction products are discussed in relation to the mutagenic activity of hydroxylamine, and some model experiments are proposed for testing the relative mutagenic roles of the exchange and addition reactions.

REFERENCES

[1] Bautz-Freese E. & Freese E. - *Proc. Natl. Acad. Sci.* **52**, 1289, 1964.
[2] Brown D. M. & Schell P. - *J. Mol. Biol.* **3**, 709, 1961.
[3] Brown D. M. & Schell P. - *J. Chem. Soc.* 208, 1965.
[4] Champe S. P. & Benzer S. - *Proc. Natl. Acad. Sci.* **48**, 532, 1962.
[5] Fikus M., Wierzchowski K. L. & Shugar D. - *Photochem. Photobiol.* **1**, 325, 1962.
[6] Fox J. J., Van Praag D., Wempen I., Doer I. L., Bendich A. & Brown C. B. - *J. Amer. Chem. Soc.* **81**, 178, 1959.
[7] Freese E., Bautz-Freese E. & Bautz E. - *J. Mol. Biol.* **3**, 133, 1961.
[8] Freese E., Bautz E. & Bautz-Freese E. - *Proc. Natl. Acad. Sci.* **47**, 845, 1961.
[9] Freese E. & Strack H. B. - *Proc. Natl. Acad. Sci.* **48**, 1796, 1962.
[10] Hilbert G. E. & Jansen E. F. - *J. Amer. Chem. Soc.* **57**, 552, 1935.
[11] Hilbert G. E. & Johnson T. B. - *J. Amer. Chem. Soc.* **52**, 1152, 1930.
[12] Janion C. & Shugar D. - *Acta Biochim. Polon.* **7**, 309, 1960.
[13] Janion C. & Shugar D. - *Biochem. Biophys. Res. Commun.* **18**, 617, 1965.
[14] Moore A. M. - *Canad. J. Chem.* **41**, 1937, 1963.
[15] Ono J., Wilson R. & Grossman L. - *J. Mol. Biol.* **11**, 600, 1965.
[16] Schuster H. - *J. Mol. Biol.* **3**, 447, 1961.
[17] Schuster H. - *Virology* **19**, 421, 1963.
[18] Sinsheimer R. L. - *Radiat. Res.* **6**, 121, 1957.
[19] Shugar D., in *The Nucleic Acids* (E. Chargaff & J. N. Davidson, eds.) Acad. Press N.Y., 1960, p. 39; see also: McLaren A. D. & Shugar D., in *Photochemistry of Proteins & Nucleic Acids*, p. 198. Pergamon Press, Oxford 1964.
[20] Shugar D. & Wierzchowski K. L. - *Biochim. Biophys. Acta*, **25**, 355, 1957.
[21] Tessman I., Poddar R. K. & Kumar S. - *J. Mol. Biol.* **9**, 352, 1964.
[22] Troll W., Belman S. & Levine E. - *Cancer Res.* **23**, 841, 1963.
[23] Verwoerd D. W., Kohlhage H. & Zillig W. - Nature **192**, 1038, 1961.
[24] Wierzchowski K. L. & Shugar D. - *Acta Biochim. Polon.* **7**, 377, 1960.

MUTAGENNA AKTYWNOŚĆ HYDROKSYLAMINY: REAKCJA Z ANALOGAMI CYTOZYNY, 5(6)-PODSTAWIONYMI ZWIĄZKAMI CYTOZYNY I NIEKTÓRYMI 2-KETO-4-ETOKSYPIRYMIDYNAMI

Streszczenie

Przeprowadzono badania nad działaniem hydroksylaminy w wodnym neutralnym środowisku na cytozynę, 5-podstawione związki cytozyny, ich glikozydy, związki cytozyny alkilowane w grupie aminowej i na związek modelowy 2-keto-4-etoksypirymidynę.

Działanie hydroksylaminy na cytozynę polega na addycji tego związku do wiązania 5,6 i natychmiastowym zastąpieniu grupy aminowej drugą cząsteczką hydroksylaminy. W środowisku kwaśnym, cząsteczka hydroksylaminy z wiązania 5,6 ulega eliminacji.

U związków grupy cytozyny z podstawnikiem w pozycji C_5 i ich glikozydów, addycja do wiązania 5,6 nie następuje, istnieje jedynie wymiana grupy aminowej na hydroksylaminową. Reakcja ta jest całkowicie ilościowa. Tak samo zachowują się 6-podstawione cytozyny.

Rodzaj zachodzących reakcji badano używając szeregu analogów 4-etoksy-2-ketopirymidyn reagujących z hydroksylaminą.

W związku z mutagenną aktywnością hydroksylaminy przedyskutowano własności produktów reakcji cytozyny i 5-metylocytozyny oraz zaproponowano doświadczenia dla wypróbowania mutagennej roli reakcji wymiany i addycji.

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DIPEPTIDE NITRILES AS SUBSTRATES FOR COLORIMETRIC DETERMINATION OF AMINOPEPTIDASES

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The purpose of the present work was to obtain some structural analogues of the natural dipeptides and to use them as substrates for determination of activity and properties of aminopeptidases.

MATERIALS AND METHODS

Substrates for synthesis of dipeptide nitriles. Phthaloylglycine, phthaloyl-DL--alanine, phthaloyl-DL-phenylalanine and phthaloyl-L-leucine were prepared after Billman & Hartig [5]. DL-Alanylnitrile was prepared according to the method of Cook & Levy [7] while glycylnitrile was obtained after Bakszejew & Gawriłow [1]. The synthesized nitriles of dipeptides were isolated as neutral salts of oxalic acid; for enzymic assays oxalate was removed by addition of an equivalent amount of calcium acetate.

L-Leucinamide·HCl was prepared after Smith & Slonim [19] and α-(*N*-γ--DL-glutamyl)-alanylnitrile after Szewczuk [24]. L-Leucyl-β-naphthylamide was obtained from Sigma Co. (St. Louis, Mo., U.S.A.) and DL-alanyl-β-naphthylamide from Schuchardt Co. (München, Germany).

Source of enzymes. Blood serum of healthy and sick persons and urine of healthy persons was used. Crude hog kidney extract was prepared by homogenizing 1 part of frozen tissue with 9 parts of 0.9% NaCl in a glass homogenizer at 4°; after centrifugation for 20 min. at 17 000 *g*, the sediment was discarded and the supernatant used for experiments. Leucine aminopeptidase from hog kidney was obtained from Fluka A.G. (Buchs, Switzerland).

Protein was determined by the method of Lowry *et al.* [10]. Three times recrystallized ovalbumin (Sigma Co., St. Louis, Mo., U.S.A.) was used as standard.

Determination of aminopeptidase activity. (a), Nitrile method. The method is based on colorimetric determination of amino acid nitriles in the presence of their acyl derivatives [23]; 0.25 ml. of 0.1 M-tris-HCl buffer, pH 8.0, was mixed in a conical

test tube with 0.25 ml. of enzyme solution or serum appropriately diluted with 0.9% NaCl, and 0.1 ml. of activator (3 μmoles of $MgCl_2$) or inhibitor or water. The enzymic reaction was initiated at 37° by addition of 0.1 ml. of 0.05 M-dipeptide nitrile acetate. The time of incubation was 30 min., and for serum 120 min. The reaction was stopped by addition of 1.3 ml. of 10% trichloroacetic acid. The suspension was clarified by centrifugation and the free amino acid nitrile was determined colorimetrically in 1 ml. of the supernatant by the previously described method [24].

(b), β-Naphthylamide method. The enzymic reaction was carried out in the same way as in the nitrile method except that the pH was 7.0 and 0.01 M-L-leucyl--β-naphthylamide·HCl or DL-alanyl-β-naphthylamide was used as substrate. Released β-naphthylamide was measured colorimetrically in 1 ml. of the supernatant by the method of Bratton & Marshall [6] in the modification of Goldbarg & Rutenburg [9].

Leucine aminopeptidase activity with leucinamide as substrate was determined by the titrimetric method of Smith [18].

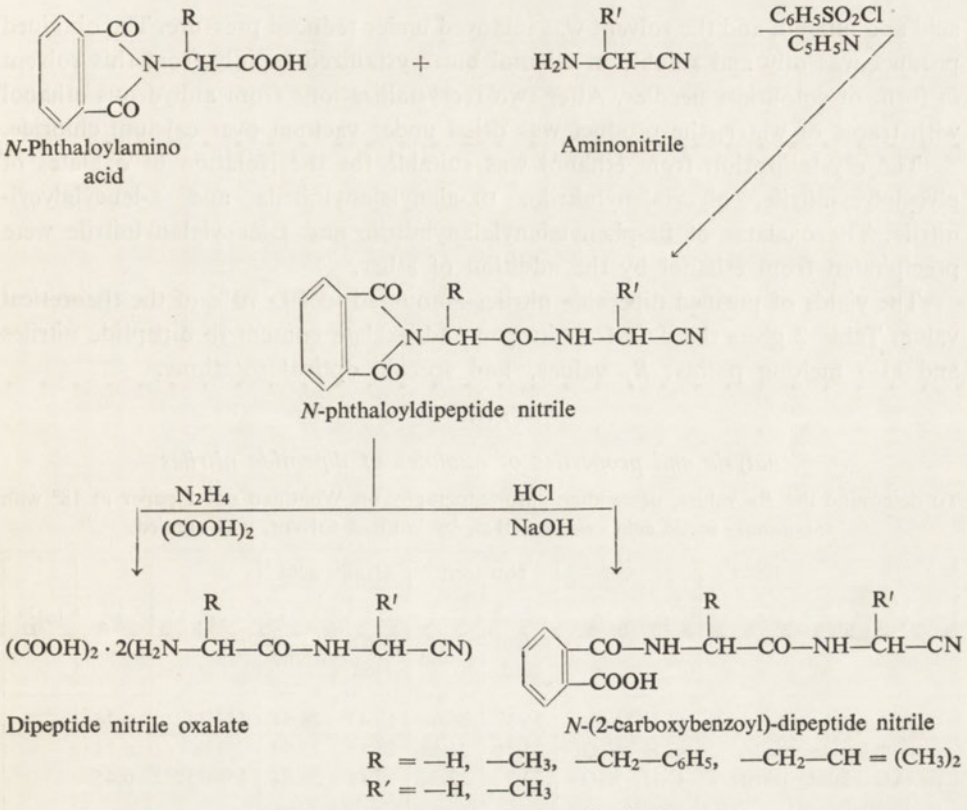
Enzymic activity was expressed in units defined as μmoles of amino acid nitrile or naphthylamide released within 1 min. of incubation by 1000 ml. of serum or 1 mg. of protein in kidney extracts and leucine aminopeptidase preparation.

Synthesis of dipeptide nitriles

The course of the two-stage synthesis of dipeptide nitriles is shown in scheme 1. The first stage, the condensation of *N*-phthaloylamino acid with aminonitrile, occurred under conditions described for the synthesis of dipeptides [21]. At the next stage the phthaloyl group was removed from *N*-phthaloyldipeptide nitrile with hydrazine and the product was isolated as a neutral oxalate salt. All dipeptide nitriles and their oxalate salts are highly soluble in water. *N*-(2-Carboxybenzoyl)--L-leucylalanylnitrile was obtained by mild alkaline hydrolysis of *N*-phthaloyl--L-leucylalanylnitrile.

Synthesis of N-*phthaloyldipeptide nitriles.* To 0.05 mole of *N*-phthaloylamino acid dissolved in 50 ml. of anhydrous pyridine, 0.05 mole (6.4 ml.) of freshly distilled benzenesulphonylchloride was added dropwise at 0 - 4° during 10 min. with constant stirring. After the next 10 min. of mixing, 0.05 mole of the appropriate freshly distilled aminonitrile was added dropwise and the stirring was continued for 30 min. The resulting mixture was left overnight at room temperature. Afterwards the mixture was added dropwise to water containing ice (1.5 kg.) and the precipitated product was collected on filter paper, recrystallized twice from 20% ethanol, and the crystalline compound dried in vacuum over calcium chloride. The yield was 60 - 70% in relation to the amount of phthaloylamino acid used. Table 1 presents the results of nitrogen determination and some physical properties of *N*-phthaloyldipeptide nitriles obtained.

Synthesis of dipeptide nitriles. To 0.03 mole of *N*-phthaloyldipeptide nitrile dissolved in 60 ml. of 96% ethanol, 5.6 ml. (0.035 mole) of 20% aqueous hydrazine



Scheme 1. Synthesis of dipeptide nitriles.

was added and the mixture was boiled under reflux for 2.5-3 hr. After cooling to 4° the precipitated phthaloylhydrazide was removed by filtration and the clear solution was concentrated at 40 - 45° under vacuum. The semi-solid product thus obtained was dissolved in 20 ml. of water and stirred for 2 hr. with 0.03 mole of freshly prepared silver oxide. The precipitate was removed by filtration and washed with water. From the combined solutions silver was removed by hydrogen sulphide and filtration. The aerated and clarified solution was brought to pH 5.6 with oxalic

Table 1

Analysis and properties of the synthesized N-*phthaloyldipeptide nitriles*

Compound	Formula	Nitrogen (%)		M.p.	$[\alpha]_D^{20}$
		calculated	found		
N-Phthaloylglycylglycylnitrile	$C_{12}H_9N_3O_3$	17.28	17.22	238-239°	
N-Phthaloylglycylalanylnitrile	$C_{13}H_{11}N_3O_3$	16.33	16.15	214-216°	
N-Phthaloyl-DL-alanylalanylnitrile	$C_{14}H_{13}N_3O_3$	15.49	15.20	133-134°	
N-Phthaloyl-DL-phenylalanyl-alanylnitrile	$C_{20}H_{17}N_3O_3$	12.10	12.41	173-175°	
N-Phthaloyl-L-leucylglycylnitrile	$C_{16}H_{17}N_3O_3$	14.04	13.72	123-125°	-9°
N-Phthaloyl-L-leucylalanylnitrile	$C_{17}H_{19}N_3O_3$	13.41	13.30	142-144°	+4°

acid and filtered, and the solvent was removed under reduced pressure. The obtained product was oily and soluble in ethanol but crystallized rapidly from this solvent in form of colourless needles. After two recrystallizations from anhydrous ethanol with traces of water, the product was dried under vacuum over calcium chloride.

The crystallization from ethanol was suitable for the isolation of oxalates of glycylglycylnitrile, glycylalanylnitrile, DL-alanylalanylnitrile and L-leucylglycylnitrile. The oxalates of DL-phenylalanylalanylnitrile and L-leucylalanylnitrile were precipitated from ethanol by the addition of ether.

The yields of purified dipeptide nitriles amounted to 50 - 70% of the theoretical value. Table 2 gives the data for nitrogen and oxalate content in dipeptide nitriles and also melting points, R_F values, and specific optical rotations.

Table 2

Analysis and properties of oxalates of dipeptide nitriles

To determine the R_F values, descending chromatography on Whatman no. 1 paper at 18° with *n*-butanol - acetic acid - water (4:1:5, by vol.) as solvent, was applied.

Compound	Formula	Nitrogen (%)		Oxalic acid (%)		M.p.	R_F	$[\alpha]_D^{20}$
		calculated	found	calculated	found			
Glycylglycylnitrile	$C_5H_8N_3O_3$	26.57	26.66	28.47	28.54	153-154°	0.26	
Glycylalanylnitrile	$C_6H_{10}N_3O_3$	24.41	24.53	26.15	25.90	138°	0.34	
DL-Alanylalanylnitrile	$C_7H_{12}N_3O_3$	22.57	22.74	24.18	24.04	158-159°	0.45	
DL-Phenylalanyl-alanylnitrile	$C_{13}H_{16}N_3O_3$	16.02	15.73	17.16	16.80	75-82°	0.71	
L-Leucylalanyl-nitrile	$C_{10}H_{18}N_3O_3$	18.41	18.35	19.72	20.08	75-82°	0.74	+20°
L-Leucylglycylnitrile	$C_9H_{16}N_3O_3$	19.62	19.89	21.01	21.24	165°	0.67	+13°

Synthesis of N-(*carboxybenzoyl*)-*leucylalanylnitrile*. Three m-moles (940 mg.) of *N*-phthaloyl-L-leucylalanylnitrile was mixed with 35 ml. of 0.1 N-NaOH at 30° until completely dissolved (about 30 min.), then the product was precipitated by adding 2 ml. of 2 N-HCl and recrystallized from 20% ethanol. The yield was 50%. The product was slightly soluble in water but dissolved readily in ethanol. Melting point 117°. Found C, 58.32; H, 6.74; N, 12.08; calculated for $C_{17}H_{21}O_4N_3 \cdot H_2O$: C, 58.44; H, 6.64; N, 12%.

Enzymic assays

It has been observed that dipeptide nitriles were enzymically hydrolysed with release of nitrile amino acids during incubation with human blood serum and urine, hog kidney homogenate and purified leucine aminopeptidase preparation. The incubations were carried out using acetate salts of dipeptide nitriles as it has been observed that, when serum was used, these substances in the oxalate form gave results lower by 20 or 30% than in the acetate form.

It has been noted that serum aminopeptidase did not split off nitrile amino acids either from the nitrile *N*-phthaloyl dipeptides or from *N*-(2-carboxybenzoyl)--L-leucylalanylnitrile.

Aminopeptidase activities of human serum. The optimum substrate concentration for 15 μl. of undiluted serum in 0.7 ml. of the incubation mixture was found to be about 4 μmoles for L-leucylalanylnitrile and L-leucylglycylnitrile, and above 10 μmoles for DL-alanylalanylnitrile, glycylglycylnitrile, and glycylalanylnitrile. For DL-phenylalanylalanylnitrile an intermediate value was obtained. The optimum concentration of L-leucyl-β-naphthylamide was about 0.5 μmole, and of DL-alanyl--β-naphthylamide about 1 μmole. In all further experiments the nitrile dipeptides were used at the concentration of 5 μmoles/0.7 ml. of the reaction mixture, and the naphthylamides at 1 μmole.

To test the sensitivity of the nitrile method, the relation between the extinctions determined by the nitrile and naphthylamide methods, and the amounts of serum was compared (Fig. 1). Over the range 2.5 - 15 μl. of serum this function was linear.

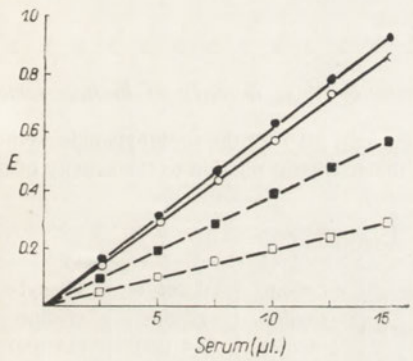


Fig. 1

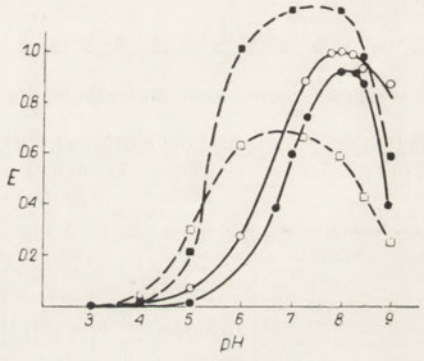


Fig. 2

Fig. 1. The effect of varying amounts of human serum on aminopeptidase activity. The activity was determined: (———), by the nitrile method, and (— — —), by the β-naphthylamide method. (○), L-Leucylalanylnitrile; (●), DL-alanylalanylnitrile; (□), L-leucyl-β-naphthylamide; (■), DL-alanyl-β-naphthylamide.

Fig. 2. The effect of pH on aminopeptidase activity in human serum. The activity was determined: (———), by the nitrile method in 16 μl. of serum, and (— — —), by the β-naphthylamide method in 32 μl. of serum. (○), L-leucylalanylnitrile; (●), DL-alanylalanylnitrile; (□), L-leucyl-β-naphthylamide; (■), DL-alanyl-β-naphthylamide.

By the nitrile method three times higher extinction was observed for L-leucylalanylnitrile than by the naphthylamide method for L-leucyl-β-naphthylamide. In experiments with DL-alanylalanylnitrile the extinction was 65% higher than with DL-alanyl-β-naphthylamide. It should be noted that the applied amount of DL--alanylalanylnitrile (5 μmoles/0.7 ml.) was below its optimum. Using standard substances (nitriles of glycine, alanine and β-naphthylamine) it has been found that the nitrile method is twice as sensitive as the naphthylamide method. In the nitrile

method, the relation of extinction to the time of enzymic reaction was linear over the range 30 - 150 min.

The optimum pH values for the activity of serum aminopeptidase toward leucyl- and alanylnitrile were determined and compared with the activity toward the corresponding naphthylamides (Fig. 2). The optimum was at pH 8.0 for L--leucylalanylnitrile and DL-alanylalanylnitrile whereas for L-leucyl- or DL-alanyl--β-naphthylamide a broad optimum was observed at pH about 7.

The effect of L-leucinamide, leucylglycine, glycylglycine, Mn^{2+} and Co^{2+} on the activity of serum peptidase toward dipeptide nitriles, L-leucyl- and DL-alanylnaphthylamides was examined (Table 3). Co^{2+} inhibited the hydrolysis of dipeptide nitriles with the exception of L-leucylalanylnitrile and activated the hydrolysis of the naphthylamides tested. The remaining peptides and Mn^{2+} inhibited the enzymic hydrolysis of all substrates tested, the inhibitory effect being stronger with nitriles than with β-naphthylamides; the inhibition was the strongest with eucylglycine and leucinamide, and very weak with glycylglycine.

Table 3

The effect of metal ions and dipeptides on aminopeptidase activity of human serum

Composition of the incubation mixture as described in the text; pH 7 for the naphthylamide method and pH 8 for the nitrile method. The figures represent the activity in relation to the activity of the control taken as 100.

Substrate	Addition					
	None (control)	Mn^{2+} (1.4 μM)	Co^{2+} (1.4 μM)	L-Leucinamide (14 μM)	L-Leucylglycine (14 μM)	Glycylglycine (14 μM)
L-Leucylalanylnitrile	100	77	104	36	24	86
L-Leucyl-β--naphthylamide	100	86	159	88	59	99
DL-Alanylalanylnitrile	100	49	34	18	5	69
DL-Alanyl-β--naphthylamide	100	80	148	68	29	99
DL-Phenylalanylalanylnitrile	100	47	31	25	8	91
Glycylalanylnitrile	100	36	43	11	6	91

Normal and pathological serum. Aminopeptidase activity in sera of healthy persons was determined with different dipeptide nitriles. Mean values for 7 men and 3 women are presented in Table 4. The hydrolysis was most rapid when DL--alanylalanylnitrile and L-leucylalanylnitrile were used as substrates. DL-Phenyl-

alanylalanylnitrile and DL-glycylalanylnitrile were hydrolysed slower. In the majority of determinations L-leucylglycylnitrile was hydrolysed slower than L-leucylalanylnitrile, and glycylglycylnitrile slower than glycylalanylnitrile.

Aminopeptidase activity toward L-leucylalanylnitrile and DL-alanylalanylnitrile was determined also in pathological sera (Table 4). An increase in enzymic activity was observed in kidney and pancreas disturbances and in neoplastic diseases.

Table 4

Aminopeptidase activity in normal and pathological serum determined with dipeptide nitriles

For conditions see text. Enzymic activity is expressed in units defined as μmoles of amino acid nitrile released during 1 min. of incubation by 1000 ml. of serum. For normal serum, mean values for 10 subjects, ±S.D, are given.

Serum	Substrate			
	L-Leucylalanyl-nitrile	DL-Alanylalanyl-nitrile	DL-Phenylalanyl-alanylnitrile	Glycylglycyl-nitrile
Normal	46.6±10	49.2±9.1	20.7±4.9	8.3±1
Carcinoma prostatae cum metastases	160	144	—	—
Cholelithiasis, Icterus mechanicus	128	122	—	—
Cirrhosis hepatis	105	103	—	—
Pancreatitis	91	68	—	—
Pancreatitis	121	128	—	—
Carcinoma cardiae	36	34	—	—
Tumor abdominis	46	48	—	—
Carcinoma ventriculi	46	46	—	—

Activity of the crude hog kidney extract and purified leucine aminopeptidase. The effect of pH on aminopeptidase activity toward various substrates is presented in Table 5. Only when *N*-leucyldipeptide nitriles were used as substrates did the changes in the pH value affect in the same way the activity of both enzymic preparations. Decomposition of β-naphthylamides by the kidney extract had a much broader pH optimum than the decomposition of the corresponding dipeptide nitriles.

The rate of hydrolysis of various substrates by the crude hog kidney extract and purified leucine aminopeptidase preparation is presented in Table 6. For either preparation, the activities are expressed in relation to the activity toward L-leucylalanylnitrile taken as 100. In both preparations, the rate of hydrolysis in this relation was the same for leucinamide, *N*-leucyldipeptide nitriles and phenylalanylalanyl-

Table 5

Effect of pH *on aminopeptidase activity in hog kidney extract and purified leucine aminopeptidase preparation (LAP)*

For conditions see text. The figures represent the activity in relation to the activity at pH 8 taken as 100.

Substrate	Source of enzyme	pH 7	pH 8	pH 9
L-Leucylalanylnitrile	extract	11	100	160
	LAP	0.2	100	140
L-Leucylglycylnitrile	extract	15	100	147
	LAP	1	100	132
L-Leucyl-β-naphthylamide	extract	100	100	89
	LAP	1	100	126
DL-Alanylalanylnitrile	extract	56	100	53
	LAP	23	100	219
DL-Alanyl-β-naphthylamide	extract	97	100	40
	LAP	0	100	200

Table 6

Aminopeptidase activity of purified leucine aminopeptidase and hog kidney extract

For conditions see text; pH 7 for the naphthylamide method and pH 8 for the nitrile method. The activity is expressed for either enzymic preparation in relation to the activity toward L-leucylalanylnitrile taken as 100.

Substrate	Crude extract	Leucine aminopeptidase
L-Leucylalanylnitrile	100	100
L-Leucinamide	1190	1130
L-Leucylglycylnitrile	110	110
DL-Phenylalanylalanylnitrile	18	18
DL-Alanylalanylnitrile	20	1
Glycylglycylnitrile	11	0.6
Glycylalanylnitrile	4	0.3
α-(*N*-γ-DL-Glutamyl)-alanylnitrile	0.3	traces
L-Leucyl-β-naphthylamide	18	5.2
DL-Alanyl-β-naphthylamide	28	traces

nitrile. The dipeptide nitriles of alanine, glycine and glutamic acid as well as alanyl--β-naphthylamide and L-leucyl-β-naphthylamide were hydrolysed only by the crude extract and were practically unaffected by leucine aminopeptidase.

DISCUSSION

Six new dipeptide nitriles, their six phthaloyl and one carboxybenzoyl derivatives were obtained by chemical synthesis. From these compounds, only *N*-phthaloylglycylglycylnitrile and *N*-phthaloylglycylalanylnitrile were obtained by Balog *et al.* [2] but in a different way. The two-stage method of dipeptide nitrile synthesis presented here is very simple and most probably does not cause racemization of amino acids, as indicated by the optical activity of the obtained *N*-leucyl dipeptidenitriles.

It is supposed that *N*-phthaloyldipeptide nitriles can be transformed by gentle hydrolysis into *N*-carboxybenzoyl derivatives in a way similar to that leading to *N*-(2-carboxybenzoyl)-leucylalanylnitrile.

The dipeptide nitriles appeared to be suitable substrates for colorimetric determination of aminopeptidase. They were all split by enzymes of human serum and urine and by hog kidney extract. One of the advantages of the presented method is its high sensitivity. By this method, even 0.2 μg. of liberated alanylnitrile [23, 24] can be determined. Another advantage is the fact that the structure of the synthetic substrates is very similar to the structure of natural dipeptides. This suggests that dipeptide nitriles may be better substrates for differentiation of aminopeptidases than the amino acid-β-naphthylamides.

During recent years, data were presented indicating the existence of several aminopeptidases [20, 13, 11, 26, 3] differing in specificity; in serum, the activity of an enzyme hydrolysing L-leucyl-β-naphthylamide was separated from that of an enzyme hydrolysing alanyl-β-naphthylamide [8, 4]. Moreover, Patterson *et al.* [16, 15] as well as other workers [25, 14, 12] presented evidence that the enzyme hydrolysing L-leucyl-β-naphthylamide is not identical with the well-known leucine aminopeptidase hydrolysing leucinamide or leucylglycine. This was demonstrated on serum, kidney and other tissues [17].

Our results are in agreement with these data. We have found that the relation of activity toward leucinamide and leucyl-β-naphthylamide was much higher for leucine aminopeptidase than for the homogenate. For both enzymic preparations, however, the relation of aminopeptidase activity toward leucinamide to the activity toward leucylalanylnitrile or leucylglycylnitrile was the same.

In our experiments, hydrolysis of leucyl-β-naphthylamide by kidney homogenate was not dependent on the pH value within the range from pH 7 to 9. However, decomposition of *N*-terminal leucine dipeptides strongly increased with the increase of pH, similarly as it was demonstrated by Smith & Spackman [20] for hydrolysis of leucinamide by leucine aminopeptidase from hog kidney. We suppose that L-leucylalanylnitrile and L-leucylglycylnitrile are hydrolysed by the same enzyme as leucinamide.

The presented results as well as the data of other authors confirm the specificity of leucine aminopeptidase which does not attack at all, or attacks very slowly, the substrates containing alanine or glycine as *N*-terminal residue.

We believe that the synthesized dipeptide nitriles containing *N*-terminal alanine

or glycine residues can be useful as substrates for colorimetric determination of alanine aminopeptidase or other aminopeptidases different from leucine aminopeptidase.

SUMMARY

1. Six new dipeptide nitriles, their phthaloyl derivatives, and *N*-(carboxybenzoyl)-leucylalanylnitrile were synthesized.

2. The dipeptide nitriles can be used as substrates for colorimetric determination of aminopeptidase activity in blood serum, urine and tissue extract.

3. The influence of metal ions, peptides and pH on aminopeptidase activity of human blood serum, hog kidney extract and purified leucine aminopeptidase was studied.

4. Aminopeptidase activity toward the dipeptide nitriles was determined in blood serum of normal and sick persons.

5. The results concerning the enzymic activity towards various dipeptide nitriles and leucyl-β-naphthylamide seem to indicate that compounds of these two types may be suitable substrates for differentiation of aminopeptidases.

REFERENCES

[1] Bakszejew A. N. & Gawriłow N. J. - *Ż. Obszcz. Chim.* **22**, 2021, 1952.
[2] Balog A., Vargha J. & Balazs I., *Studia univ. Victor Babes et Bolyai*, 3, No. 4, Ser. 1, No. 2, 175, 1958.
[3] Behal F. J., Hamilton R. D., Davson F. B. & Terrell L. C. - *Arch. Biochem. Biophys.* **108**, 207, 1964.
[4] Behal F. J., Hamilton R. D., Kanavage C. B. & Kelly E. C. - *Arch. Biochem. Biophys.* **100**, 308, 1963.
[5] Billman J. H. & Hartig W. F. - *J. Amer. Chem. Soc.* **70**, 1473, 1948.
[6] Bratton A. C. & Marshall E. K. Jr. - *J. Biol. Chem.* **128**, 537, 1939.
[7] Cook A. H. & Levy A. L. - *J. Chem. Soc.* 643, 1950.
[8] Dubbs C. A., Vironia Ch. & Hilburn J. M. - *Nature* **191**, 1204, 1961.
[9] Goldbarg J. A. & Rutenburg A. M. - *Cancer* **11**, 283, 1958.
[10] Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. - *J. Biol. Chem.* **193**, 265, 1951.
[11] Matheson A. T., Bjerre S. & Hanes C. S. - *Can. J. Biochem. Physiol.* **41**, 1741, 1963.
[12] McDonald J. K., Reilly T. J. & Ellis S. - *Biochem. Biophys. Res. Commun.* **16**, 135, 1964.
[13] Nachlas M. M., Goldstein Th. P. & Seligman A. M. - *Arch. Biochem. Biophys.* **97**, 223, 1962.
[14] O'Connell W. & Winzler R. J. - *Cancer Res.* **23**, 78, 1963.
[15] Patterson E. K., Hsiao S. H. & Keppel A. - *J. Biol. Chem.* **238**, 3611, 1963.
[16] Patterson E. K., Keppel A. & Hsiao S. H. - *J. Histochem. Cytochem.* **9**, 609, 1961.
[17] Shoichi N. & Hitoshi T. - *Clin. Chim. Acta* **10**, 572, 1964.
[18] Smith E. L., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 2, p. 88. Academic Press, New York 1955.
[19] Smith E. L. & Slonim M. B. - *J. Biol. Chem.* **176**, 835, 1948.
[20] Smith E. L. & Spackman D. H. - *J. Biol. Chem.* **212**, 271, 1955.
[21] Sokołowska T., Kupryszewski G. & Taschner E. - *Roczniki Chemii* **32**, 813, 1958.
[22] Szewczuk A. - *Roczniki Chemii* **32**, 131, 1958.

[23] Szewczuk A. - *Chemia Analityczna* **4**, 971, 1959.
[24] Szewczuk A. & Orłowski M. - *Clin. Chim. Acta* **5**, 680, 1960.
[25] Thompson D. P. & Schwartz R. B. - *Clin. Res.* **7**, 393, 1959.
[26] Wolff J. B. & Resnik R. A. - *Biochim. Biophys. Acta* **73**, 613, 1963.

KOLORYMETRYCZNE OZNACZANIE AMINOPEPTYDAZ PRZY UŻYCIU NITRYLI DWUPEPTYDÓW JAKO SUBSTRATÓW

Streszczenie

1. Otrzymano sześć nitryli dwupeptydów, sześć ich ftaloilowych pochodnych oraz *N*-(karboksybenzoilo)-leucyloalanylonitryl.

2. Nitryle dwupeptydów można zastosować jako substraty do kolorymetrycznego oznaczania aktywności aminopeptydazowej w surowicy, moczu i ekstrakcie tkankowym.

3. Zbadano wpływ jonów metali, peptydów, pH na aktywność aminopeptydazową surowicy ludzkiej, ekstraktu z nerki świńskiej i oczyszczonej aminopeptydazy leucynowej.

4. Oznaczono aktywność aminopeptydazową surowicy ludzi zdrowych i chorych.

5. Wyniki aktywności enzymatycznej względem różnych dwupeptydów nitryli i leucylo-β-naftylamidu pozwalają przypuszczać, że związki obu typów nadają się do różnicowania aminopeptydaz.

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OXYGEN UPTAKE AND AMMONIA RELEASE IN NORMAL AND γ-IRRADIATED EGGS OF *BOMBYX MORI*

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Previously it has been reported [13] that the hatching of larvae was inhibited when 20 kr. of γ-rays was applied on the eggs of *Bombyx mori* at diapause as well as just before or at the beginning of postdiapausal development. The loss of hatchability, however, does not foreclose the immediate death of embryo and the life span of irradiated embryo may vary under different conditions.

It is known that the effect of ionizing radiation upon different fundamental processes occurring during the embryonal life is not the same. Hubert has shown already in 1929 [12] that a dose of X-rays stopping entirely the growth of chick embryo reduced the glycolysis by 45% and the respiration only by 15%. The relative radioresistance of the oxygen uptake process was also confirmed in embryos of insects [16]. Thus the inhibition of oxygen uptake would denote the upper limit of radiation-damaged embryonal development at least as far as the energy-requiring processes are concerned.

In this paper are presented the results of studies on the effect of the dose of γ-rays, of the time-interval between the irradiation and the onset of postdiapausal development as well as of the temperature at which this development occurred, upon the oxygen uptake in the eggs of *B. mori*.

The release of gaseous ammonia was also examined because Ashbel [2] reported this process to operate at a relatively high rate in the eggs of *B. mori*.

MATERIAL

The eggs of *B. mori* were purchased from an industrial culture. Two monovoltine races were studied: "warska" and white cross-bred designated in Milanówek as (Aa×Ia)×(Dg×Ea). The eggs were stored for about 120 days at 15° and thereafter for at least 100 days at 4°.

Single doses of 20 kr. or 200 kr. of γ-rays were delivered from a ^{60}Co source (about 45 curie) at a rate of about 200 r/min. The irradiation was performed at the beginning of storage at 4° (mid-diapause) or towards the end of storage at this

temperature (postdiapausal period, when the arrest of development is due solely to the low temperature). Thus the eggs irradiated at the same stage of embryogenesis (blastula) were kept at 4° for three months or for seven days before the storage temperature was raised above the treshold for embryogenesis. The final temperature of incubation was attained gradually within four days, simultaneously for the irradiated and control samples of eggs. Massive hatching of larvae occurred usually after 10 - 11 days of incubation at 25° and after 8 days of incubation at 30°.

METHODS

The oxygen uptake was measured by the manometric method [18] in air. The flasks contained: 0.2 ml. 20% KOH in the center well, 100 - 200 mg. of whole eggs in the side arm and, to absorb the released ammonia, 3 ml. of 0.02 N-H_2SO_4 in the main compartment. Equilibration of temperature was attained after 30 min. of shaking with opened stopcocks. The manometer readings were made every 30 min. during 4 hr. For each experiment a new portion of eggs was used.

The absorbed ammonia was estimated colorimetrically by nesslerization against a blank run simultaneously in each experiment.

RESULTS

The influence of irradiation. The effect of the dose of γ-rays on the respiration was studied in the eggs of warska race irradiated at the postdiapausal period. The uptake of oxygen was measured at 25° regardless of the temperature of storage.

In the normal eggs the uptake of O_2 at diapause (storage temperature 4°) was 37 µl/g./hr.$\pm$11 (S.D). When the storage temperature was gradually raised to 25° the oxygen uptake amounted to 81$\pm$16 µl./g./hr. in control and to 88$\pm$13 µl./g./hr. in the irradiated eggs. Thus no differences were found at this period between the normal eggs and those irradiated with 20 kr. or 200 kr.

However, during incubation at 25° marked differences became evident (Fig. 1). A gradual increase in the respiration rate was observed in normal eggs during the whole period of development till the hatching. A few hours before hatching the oxygen uptake amounted to about 750 µl./g./hr. indicating a 20-fold increase over the diapausal value.

In the eggs irradiated with 20 kr. the gradual increase in the respiration rate during the incubation was also observed. The actual rate was, however, always much lower than that in normal eggs incubated simultaneously. On the 10th day the uptake was 150 µl. O_2/g./hr., representing hardly 1/4 of the value found at this time in the normally developing control. This slow, gradual increase in the respiration rate continued till the 15th day of incubation. During further 5 days the oxygen uptake persisted at a constant level of nearly 300 µl./g./hr.

In the eggs irradiated with 200 kr. no increase in the rate of oxygen uptake was found during the incubation at 25°. The respiration continued, however, at the

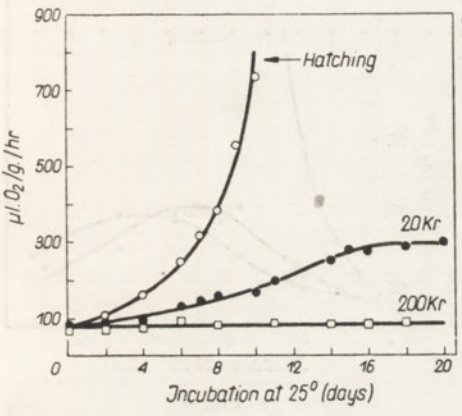

Fig. 1

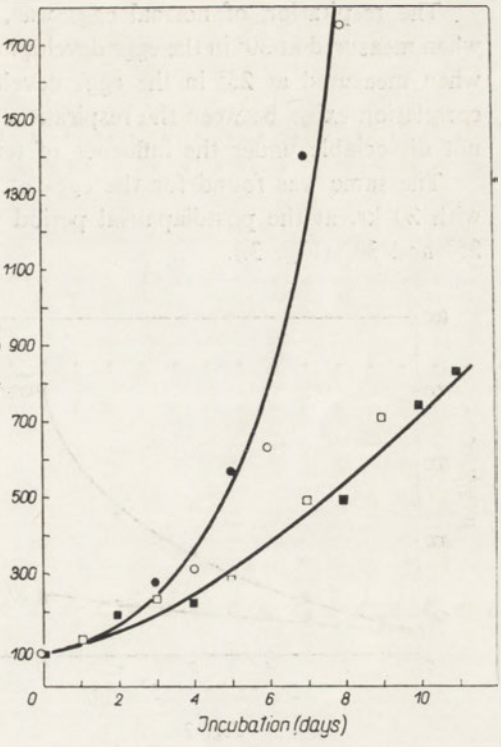

Fig. 2

Fig. 1. The influence of the dose of γ-rays on the postdiapausal respiration of *B. mori* eggs at 25°. The eggs of warska race were irradiated at the postdiapausal period of storage at 4°, stored at this temperature for 7 days after irradiation and then incubated at 25°. A typical experiment is presented. Eggs irradiated (●), with 20 kr; (□), with 200 kr; (○), control.

Fig. 2. The influence of incubation temperature on the postdiapausal respiration of normal eggs of *B. mori*. The eggs of the white cross-bred race were incubated at 25° or at 30°, and the respiration of both was measured at two temperatures. Eggs incubated at 30°, measured (●), at 30° and (○), at 25°. Eggs incubated at 25°, measured (■) at 25° and (□), at 30°.

initial rate for 18 days. It is noteworthy that the "age-dead" eggs of warska race (the eggs which have lost the ability to develop in consequence of prolonged storage at low temperature) did not take up oxygen at all while incubated at 25° for two weeks and controlled during this period.

The effect of temperature on the oxygen uptake was studied in the eggs of both races. Figure 2 presents the respiration curves characteristic for the normal eggs of white cross-bred race investigated at 25° and 30°. The increase in temperature of incubation shortened the time needed for hatching from 11 days at 25° to 8 days at 30°. The acceleration of development was accompanied by an increased respiration rate. Thus one day before hatching the oxygen uptake amounted to about 750 µl./g./hr. in the eggs developing at 25° and to about 1400 µl./g./hr. in those developing at 30°.

The respiration of normal eggs was, however, neither substantially increased when measured at 30° in the eggs developing at 25°, nor was it substantially decreased when measured at 25° in the eggs developing at 30°. This indicates that a close correlation exists between the respiration rate and the stage of development, being not dissociable under the influence of temperature.

The same was found for the eggs of warska race both normal and irradiated with 20 kr. at the postdiapausal period when incubated at 25° and examined at 25° and 30° (Fig. 3.).

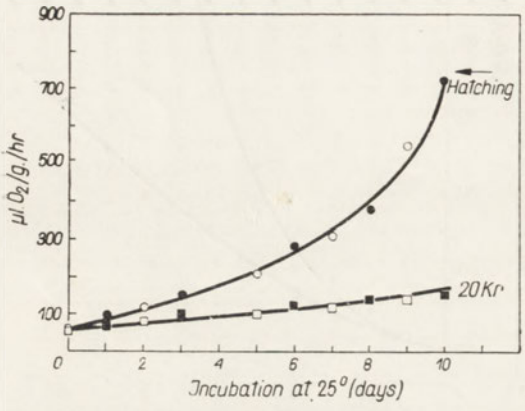

Fig. 3

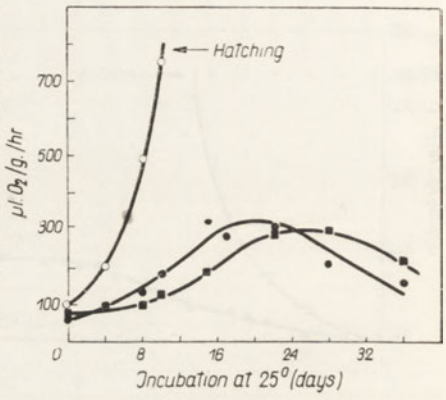

Fig. 4

Fig. 3. The independence of the respiration rate from the temperature at which the determinations were made, in normal and irradiated eggs of *B. mori*. The eggs of warska race were irradiated at the postdiapausal period of storage at 4°, stored at this temperature for 7 days and thereafter incubated at 25° simultaneously with the control sample. Normal eggs incubated at 25°, measured (●), at 25° and (○), at 30°. Eggs irradiated with 20 kr. incubated at 25°, measured (■), at 25° and (□) at 30°.

Fig. 4. The influence of time interval between irradiation and onset of postdiapausal incubation on the respiration of *B. mori* eggs at 25°. The eggs of the white cross-bred race were irradiated with 20 kr. at blastula stage, stored thereafter for 3 months or for 7 days at 4° and then incubated at 25°, simultaneously with the control sample. Eggs irradiated with 20 kr. (●), stored for 3 months at 4° and (■), stored for 7 days at 4°; (○), control.

The influence of the time interval elapsed between the irradiation and the onset of postdiapausal incubation was studied in the eggs of white cross-bred. The eggs irradiated with 20 kr and stored thereafter at 4° for 3 months (long-storage eggs) or for 7 days (short-storage eggs), were incubated at 25° or 30° and their respiration was measured at the temperature of incubation.

Figure 4 presents the respiration of irradiated and control eggs incubated at 25°. In the long-storage as well as in the short-storage eggs the maximum rate of oxygen uptake was the same and amounted to about 300 µl./g./hr. This value corresponds roughly to the rate of respiration of control eggs at the mid-period of postdiapausal development. The maximum respiration rate in the long-storage eggs was, however, attained on the 16th day while in the short-storage eggs only on the 22nd day of

incubation. The oxygen uptake continued at this maximum rate in both long- and short-storage eggs for about 5 days and thereafter decreased slowly, being on the 35th day still higher than at the beginning.

The results of analogous experiments carried out at 30° are presented in Fig. 5. The maximum rate of respiration during the incubation amounted to about 300 μl. O_2/g./hr. in the long-storage eggs and to about 250 μl. O_2/g./hr. in the short--storage eggs. This maximum was, however, attained earlier at 30° than at 25°.

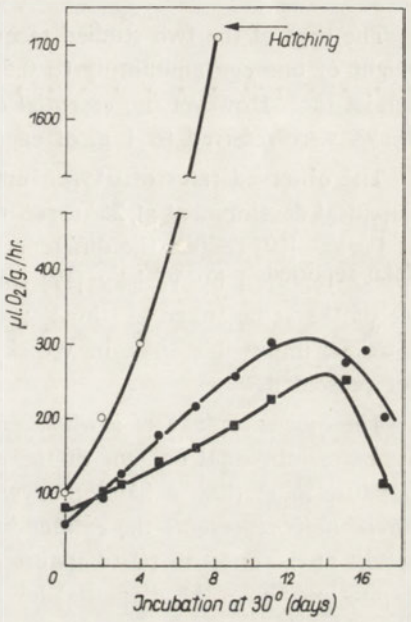

Fig. 5. The influence of the incubation temperature on the radiation-injured respiration of the eggs of *B. mori*. Material and conditions as in Fig. 4. Incubation at 30°. Eggs irradiated with 20 Kr. (●), stored for 3 months at 4° and (■), stored for 7 days at 4°; (○), control.

So the long-storage eggs respired at the maximum rate already on the 11th day, and the short-storage ones on the 14th day of incubation at 30°. This maximum rate of oxygen uptake in both long- and short-storage eggs lasted at 30° only for a short time and already on the 17th day of incubation a marked decrease was observed.

The release of gaseous ammonia. Ashbel [2] reported that the diapausing eggs of *B. mori* release as much as 0.46 g. NH_3/kg./24 hr.; this would give an output of about 20 μg. NH_3/g./hr., an amount well measurable in the experimental conditions used. The release of ammonia was examined in the normal and irradiated eggs during diapause as well as during the postdiapausal incubation at 25° or 30°. Our results, however, did not substantiate the observations of Ashbel. In 85 out of 120 experiments in which absorbed ammonia was estimated, no NH_3 at all or only traces were found and in the remaining 35 experiments which gave positive results, only 10 results amounted to more than 25%, never reaching 100%, of the value reported by Ashbel. The few positive results found were in no way correlated with the period of normal or of radiation-injured embryonal development. In some

experiments performed with 1 g. of eggs in order to check the positive result noted in a 0.1 g. sample, no NH_3 or only traces were found. Thus some positive results noted may be attributed to contaminations.

DISCUSSION

Normal eggs

The eggs of the two studied races of *Bombyx mori* differed in size, the average weight of one egg amounting to 0.55 mg. in white cross-bred and to 0.75 mg. in warska race. However, no essential differences between the races existed when the results were referred to 1 g. of eggs.

The observed rates of oxygen uptake in the eggs of *B. mori* during their post-diapausal development at 25° agree reasonably well with the CO_2 output observed by Farkas [10] despite the differences in handling of the material. The rates of O_2 uptake reported by Wolsky [19] for diapausing and developing eggs of *B. mori* are of the same range as those presented here; more accurate comparison is, however, impossible since in Wolsky's paper details needed for recalculation are not presented.

The oxygen uptake of a whole egg is due to the embryonal tissue as well as to the extraembryonal content of the egg. Bodine & Boell [7] reported that during diapause more than a half of oxygen consumption of whole eggs of *Melanoplus differentialis* represents the extraembryonal respiration and that the increase in O_2 uptake at the onset of postdiapause occurs first in the yolk cells. The initial rise in respiration above the diapausal level observed in the eggs of *B. mori* on the first day of incubation may also represent the increase in extraembryonal oxygen uptake. Along with the growth of embryo at the expense of yolk, the part played by the embryonal respiration increases gradually and just before hatching the respiration of the whole egg represents that of the embryo alone. The maximum respiration rate found in the whole eggs of *B. mori* just at hatching (about 1.7 ml. O_2/g./hr. at 30°, see Fig. 2) lies within the range of values reported by Białaszewicz [5] for the first instar larvae of this insect.

The respiration in normal eggs of *B. mori* was correlated with the embryonal development since the increase in incubation temperature from 25° to 30° accelerated the development and increased markedly the respiration, whereas the rate of oxygen uptake characteristic for a given stage of embryogenesis remained unaffected when measured at temperatures differing by 5° in plus or in minus from the incubation temperature. These results are consistent with the observation of Bodine [6] that the respiration of grasshopper eggs developing at 25° did not diminish when measured after some time of storage at low temperature. So the fact that temperature does not dissociate developmental rate from respiratory rate, observed in amphibian [17] and echinoderm [3] embryos, may be valid also in insect embryo.

The influence of temperature on the postdiapausal respiration of *B. mori* eggs

became evident only on the 4th day of incubation and was already marked on the 5th day. According to the data reported by Michajłow [14] and the results of Chino [8], in the eggs of *B. mori* developing at 25° after diapause blastokinesis (revolution of embryo) does not begin before the 5th day of incubation. The moving-round embryo is already differentiated into 18 metamers. Thus the acceleration of post-diapausal embryogenesis in *B. mori* due to temperature involved mainly later stages of development, beginning with blastokinesis.

Irradiated eggs

The initial increase in respiration from the diapausal level of 37 μl. O_2/g./hr. to about 80 μl. O_2/g./hr. observed on the first day of incubation in the eggs of *B. mori* irradiated with 200 kr. may be attributed, as in normal eggs, to the enhancement of oxygen uptake in yolk. Since this initial respiration rate persisted till the 18th day of incubation, it is evident that neither immediate death of the embryo occurred nor the energy-requiring processes took place. Tahmisian [16] reported that in diapausing eggs of *Melanoplus* the respiration was reduced just after irradiation with 200 kr. of X-rays, although the embryo was still alive on the 35th day of storage at 25°. The presented results are comparable with those of Tahmisian despite the different sources of radiation used, as it has been shown [1] that in insects the biological effectiveness of X- and γ-rays is nearly the same. The apparently greater radiosensitivity of respiration of grasshopper eggs may be due to the fact that at the onset of diapause the embryonal development in grasshopper eggs was blocked at a more advanced stage than that in the eggs of *B. mori*. Thus in *Melanoplus* eggs the embryonal respiration, possibly the more sensitive one, made up a greater part of postdiapausal respiration of the whole egg.

It is interesting that even very high doses of ionizing radiation did not cause immediate death of the insect embryo. Grosch [11] reviewing the entomological aspects of radiation emphasized that at postembryonal stages of insect life the radiation death is as a rule delayed. This may be true for the embryonal life of insects, too.

The dose of 20 kr. was found to be less deleterious for the respiration of eggs than that of 200 kr. On incubation at 25° the respiration of irradiated eggs increased gradually to about 300 μl. O_2/g./hr. and then persisted at this level for some days. This maximum at which the respiration of irradiated eggs was blocked corresponds to the rate of oxygen uptake found in normally developing control on the 6th day, that is at the time when the gastrulation was accomplished and the embryo was just before or started the blastokinesis. This qualitative analogy does not necessarily mean the same energetic efficiency of respiration since it was shown in isolated mitochondria [9] that ionizing radiation results in uncoupling oxidations from phosphorylations, these latter being more sensitive. However, there is some analogy between the presented results and the observations of Neifah [15] concerning fish embryo. Neifah has found that the end of gastrulation is a critical stage at which

stopped the development of the embryo irradiated at the late blastula stage with an X-ray dose injurious for nuclei (20 kr.). Since this dose gave an identical result when delivered at the mid-gastrula stage, Neifah supposed that embryonal cells were already sufficiently provided with some nuclear factor at the late blastula stage, to develop till the end of gastrulation even when the nuclei have been injured.

The postdiapausal onset of increase in respiration of eggs irradiated with 20 kr. was markedly delayed in comparison with that of control eggs. The delay observed at 25° was about 6 days shorter when the time between the irradiation and the onset of postdiapausal development was prolonged from 1 week to 3 months. Thus a partial recovery concerning the delay but not the final effect, could be observed. The increase in temperature of incubation from 25° to 30° reduced markedly the delay in both long- and short-storage eggs, it did not alter, however, the final inhibitory effect. This would indicate that no correlation exists between the processes involved in the delay of development due to radiation and the injury responsible for the inhibition of embryogenesis at a certain stage.

The respiratory activity at 30° was blocked at the postgastrulation level in the long-storage eggs or even below this level in the short-storage eggs and decayed in both more abruptly than it was observed at 25°. This is consistent with the observations made by Baldwin [4] on adults of *Dahlbominus fuscipennis* that ionizing radiation induced a susceptibility to temperatures that were normally well tolerated by non-irradiated individuals.

SUMMARY

1. The rate of postdiapausal respiration in normal eggs of *Bombyx mori* was correlated with the developmental rate. The enhancement due to temperature involved mainly later stages, beginning with blastokinesis.

2. Radiation doses inhibiting completely hatching, did not cause immediate death of the eggs. Respiration of irradiated eggs was blocked after 200 kr. at the stage corresponding to blastula, and after 20 kr. at that corresponding to late gastrula.

3. The increase in respiration of 20 kr.-irradiated eggs was delayed. The delay, but not the final effect, depended on the temperature and on time interval between irradiation and incubation.

4. Neither in irradiated nor in normal eggs the release of NH_3 could be stated.

REFERENCES

[1] Amy R. L. - *Radiat. Res.* **3**, 166, 1955.
[2] Ashbel R. - *Boll. Soc. Ital. Biol. Sper.* **4**, 1, 1928.
[3] Atlas M. - *Physiol. Zool.* **8**, 290, 1935.
[4] Baldwin W. F. - *Radiat. Res.* **8**, 17, 1958.
[5] Białaszewicz K. - *Acta Biol. Exper.* **11**, 229, 1937.
[6] Bodine J. H. - *Physiol. Zool.* **2**, 459, 1929.

[7] Bodine J. H. & Boell E. J. - *J. Cell. Comp. Physiol.* **8**, 357, 1936.
[8] Chino H. - *J. Insect Physiol.* **6**, 231, 1961.
[9] Clarke I. D. & Lang J. - *Radiat. Res.* **24**, 142, 1965.
[10] Farkas K. - *Arch. ges. Physiol.* **98**, 490, 1903.
[11] Grosch D. S. - *Ann. Rev. Entomol.* **7**, 81, 1962.
[12] Hubert R. - *Pflueger's Arch. ges. Physiol.* **223**, 33, 1929.
[13] Lassota Z. - *Acta Biochim. Polon.* **10**, 379, 1963.
[14] Michajłow E. N., *Szołkowodstwo*. Gos. Izdat. Sielskochoz. Lit., Moskwa 1950.
[15] Neifah A. A. - *Żurn. Obszcz. Biol.* **20**, 202, 1959.
[16] Tahmisian T. N. - *J. Expt. Zool.* **112**, 449, 1949.
[17] Tyler A. - *Biol. Bull.* **71**, 82, 1936.
[18] Umbreit W. W., Burris R. H. & Stauffer J. F., *Manometric Techniques*. Burgess Publ. Co., Minneapolis 1957.
[19] Wolsky A. - *Matt. Termeszett Ertes* **159**, 893, 1940.

ZUŻYWANIE TLENU I WYDZIELANIE AMONIAKU PRZEZ NORMALNE I NAŚWIETLONE PROMIENIAMI γ JAJA *BOMBYX MORI*

Streszczenie

1. W normalnych jajach oddychanie w okresie podiapauzalnym jest zależne od szybkości rozwoju. Przyśpieszenie wywołane podwyższeniem temperatury dotyczy głównie późniejszego okresu rozwoju, po blastokinezie.

2. Dawki promieniowania hamujące całkowicie wylęg gąsienic nie powodowały natychmiastowej śmierci jaja. Naświetlanie dawką 200 kr. blokowało oddychanie na poziomie odpowiadającym stadium blastuli, a naświetlanie dawką 20 kr. — na poziomie odpowiadającym późnej gastruli.

3. Dawka 20 kr. opóźniała narastanie intensywności oddychania w okresie podiapauzalnym. Temperatura oraz długość przerwy między naświetleniem a inkubacją wpływały na opóźnienie, ale nie zmieniały końcowego efektu.

4. Nie stwierdzono wydalania amoniaku ani w jajach normalnych ani w naświetlonych.

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UNCOUPLING OF OXIDATIVE PHOSPHORYLATIONS BY BASIC PROTEINS

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A number of authors have reported an uncoupling effect of basic proteins, such as protamine [9], ribonuclease [2], histones [7], and of polylysine, a synthetic polypeptide [9], on oxidative phosphorylations in plant or animal mitochondria. Rivenbark & Hanson [9] have suggested that basic proteins act by penetrating to the negatively charged sites of mitochondria involved in the phosphorylation.

Rzeczycki *et al.* [11, 12] isolated from hog kidney mitochondria a basic protein (MBP) which inhibited electron transport through the respiratory chain [10] and swelling of mitochondria, both spontaneous and induced [3]. Therefore it seemed worthwhile to examine the effect of MBP on oxidative phosphorylation and compare it with the effect of protamine and ribonuclease.

MATERIALS AND METHODS

Rat liver mitochondria were used for experiments. Albino Wistar rats were killed by decapitation; one liver was homogenized in an Elvehjem-Potter homogenizer in a tenfold volume of 0.25 M-sucrose containing 1 mM-EDTA, pH 7.4. Mitochondria were centrifuged between 800 - 7000 *g* for 10 min., washed three times and suspended in 2.5 ml. of the same solution. The procedure was carried out at 0°. Isolated mitochondria were taken for oxidative phosphorylation measurement either fresh (non-preincubated) or after preincubation with basic protein. The preincubation was carried out at room temperature (20 - 25°) for 10 min. with basic proteins dissolved in 0.25 M-sucrose - 1 mM-EDTA solution of pH 7.4 or in the incubation mixture used for the measurement of oxidative phosphorylations, containing 40 mM-sucrose, 20 mM-tris-HCl buffer, pH 7.4; 2 mM-EDTA, pH 7.4; 15 mM-sodium phosphate buffer, pH 7.4; 3 mM-$MgSO_4$, 0.05 mM-ATP and 20 mM--glucose. One ml. of the preincubation solution contained 0.2 ml. of the suspension of mitochondria (about 8 mg. protein), and one of the basic proteins: 200 μg. of protamine or 300 μg. of MBP or 1000 μg. of ribonuclease. After 10 min. of preincubation the mitochondria were centrifuged at 10 000 *g* at 0° for 10 min., washed and resuspended in sucrose solution, the volume being adjusted so as to obtain

the same concentration of protein as in the initial suspension. Mitochondria preincubated in the same way but without the addition of basic protein, served as control.

Oxidative phosphorylation (P/O) was measured in the conventional Warburg apparatus at 25°. The main compartment of the manometric vessel contained: sucrose, 40 μmoles; tris-HCl buffer, 20 μmoles; EDTA (pH 7.4), 2 μmoles; sodium phosphate buffer (pH 7.4), 15 μmoles; $MgSO_4$, 3 μmoles; ATP, 0.05 μmole; hexokinase (containing 100 units [6]), 0.5 mg.; glucose, 20 μmoles; substrate, 15 μmoles. Three substrates were used: potassium malate with sodium pyruvate, potassium succinate, and potassium ascorbate. When ascorbate was used, cytochrome *c* was added to a final concentration of 0.2×10^{-7} M. In experiments in which non-preincubated mitochondria were to be used the basic proteins dissolved in 0.02 M-tris-HCl buffer, pH 7.4, were added in the following amounts: protamine, 200 μg.; MBP, 300 μg.; ribonuclease, 1000 μg. At zero time 0.2 ml. of the suspension in sucrose solution of mitochondria non-preincubated or preincubated with or without the addition of basic protein, was added. The final volume of the incubation mixture was 1 ml. In the central well about 50 μl. of 10% KOH solution was placed. Temperature equilibration was carried out during the first 5 min. of incubation, then oxygen uptake was measured every two minutes. The reaction was stopped one minute after the last measurement by adding 0.5 ml. of 20% trichloroacetic acid. Total time of the incubation together with the temperature equilibration period and the one minute after the last reading, varied between 12 - 46 min.

The deproteinized incubation mixture was filtered through Whatman no. 1 paper to a calibrated tube. The manometric vessel and filter paper were washed three times with water and the total made up to 10 ml. Inorganic phosphate was determined by the method of Gomori [8] and its uptake was calculated in relation to the control in which trichloroacetic acid was added at zero time.

Oxygen uptake was calculated from the values read on manometers [1] taking into account a correction for 5 min. of temperature equilibration and one minute of incubation after the last reading. The correction was determined graphically by extrapolation of the plot of manometer readings against time.

To determine protein in the suspensions of mitochondria, 0.1 ml. samples were taken, made up to 1 ml. with 0.1% sodium deoxycholate and then the biuret reagent was added [5].

Mitochondrial basic protein (MBP) was prepared from hog kidney as described previously [11, 12].

Reagents used: Ribonuclease and protamine sulphate (B.D.H., England); hexokinase, type III (Sigma, U.S.A.); cytochrome *c* (Wytwórnia Surowic i Szczepionek, Kraków, Poland); ATP, sodium salt (Tarchomińskie Zakłady Farmaceutyczne, Poland); L-malic acid (Light, England); L-ascorbic acid (Politechnika Śląska, Gliwice, Poland); $MgSO_4$ (Merck, Germany); sodium phosphate *prim.* and *sec.* (Xenon, Łódź, Poland); potassium succinate, sodium pyruvate, glucose and sucrose (Biuro Obrotu Odczynnikami, Gliwice, Poland); sucrose was deionized with Amberlite IRC-50. Water used throughout the experiments was bidistilled and passed through Amberlite IRC-50.

RESULTS

The effect of three basic proteins on oxidative phosphorylations coupled to the oxidation of malate with pyruvate, succinate and ascorbate, is presented in Tables 1 - 3 and summarized in Table 4.

In mitochondria non-preincubated with the protein the addition of MBP inhibited oxidative phosphorylation coupled to the oxidation of ascorbate by 70%. The inhibition with malate-pyruvate as substrate was 28%, and with succinate it was much smaller (only 9%). For protamine the respective values were: with ascorbate 43%, with malate-pyruvate 22%, with succinate 27%. Ribonuclease had almost no effect with the exception of phosphorylation coupled to the oxidation of ascorbate which was inhibited by 32%.

Table 1

Effect of protamine on oxidative phosphorylations in rat liver mitochondria

Oxygen uptake (μl./mg. protein/hr.) and calculated P/O ratios are given. Values are the means from two measurements. Incubation conditions: basic protein was added to non-preincubated mitochondria; mitochondria preincubated with basic protein in 0.25 M-sucrose were incubated without the addition of basic protein (details in the text).

Substrate (15 mM)	Expt. no.	Non-preincubated mitochondria				Mitochondria preincubated			
		Incubation							
		without protamine (control)		with protamine		without protamine (control)		with protamine	
		Oxygen uptake	P/O	Oxygen uptake	P/O	Oxygen uptake	P/O	Oxygen uptake	P/O
Malate+ pyruvate	1	27.8	2.27	17.0	1.95	28.0	2.07	15.4	1.54
	2	25.3	2.40	15.6	1.68	25.3	2.43	15.8	1.46
Succinate	1	24.1	1.33	15.5	1.02	37.3	1.23	36.1	0.89
	2	45.4	1.52	33.7	1.27	48.8	1.35	41.2	1.12
	3	42.2	1.84	15.7	1.04	44.5	1.57	29.9	0.86
Ascorbate	1	19.3	0.99	8.8	0.74	21.3	0.85	14.1	0.23
	2	15.3	1.02	13.4	0.67	18.5	0.66	21.5	0.30
	3	20.4	0.59	10.3	0.18	22.3	0.36	15.2	0.13

The 10 min. preincubation of mitochondria with basic protein in sucrose solution enhanced the effect of the basic protein added to the incubation mixture, and oxidative phosphorylation was inhibited to a greater extent than in the non-preincubated samples. Of the three basic proteins studied, MBP had the greatest inhibitory effect on phosphorylation coupled with the oxidation of the three substrates; the inhibition amounted with malate-pyruvate to 53%, with succinate to 67% and with ascorbate

Table 2

Effect of mitochondrial basic protein on oxidative phosphorylations in rat liver mitochondria

Conditions as for Table 1.

Substrate (15 mM)	Expt. no.	Non-preincubated mitochondria				Mitochondria preincubated			
		Incubation							
		without MBP (control)		with MBP		without MBP (control)		with MBP	
		Oxygen uptake	P/O	Oxygen uptake	P/O	Oxygen uptake	P/O	Oxygen uptake	P/O
Malate+ pyruvate	1	15.5	2.62	17.1	1.93	17.7	1.98	11.5	1.10
	2	17.4	2.67	16.3	1.86	15.5	2.55	7.6	0.97
Succinate	1	30.7	1.52	25.5	1.33	31.2	1.35	16.1	0.67
	2	63.9	1.16	36.7	1.06	61.0	1.13	26.9	0.22
	3	62.2	1.30	45.3	1.20	49.2	1.13	37.9	0.32
Ascorbate	1	23.0	0.87	13.2	0.28	21.8	0.68	12.7	0.14
	2	23.1	0.73	11.1	0.27	20.2	0.57	10.9	0.00

Table 3

Effect of ribonuclease on oxidative phosphorylations in rat liver mitochondria

Conditions as for Table 1.

Substrate (15 mM)	Expt. no.	Non-preincubated mitochondria				Mitochondria preincubated			
		Incubation							
		without ribonuclease (control)		with ribo-nuclease		without ribonuclease (control)		with ribo-nuclease	
		Oxygen uptake	P/O	Oxygen uptake	P/O	Oxygen uptake	P/O	Oxygen uptake	P/O
Malate+ pyruvate	1	26.9	2.49	28.1	2.30	25.1	2.13	23.3	2.10
	2	16.1	2.32	15.5	2.34	12.2	2.39	12.6	1.98
	3	27.4	2.30	30.1	2.02	24.7	2.19	21.2	1.84
Succinate	1	31.3	1.49	32.1	1.25	28.7	1.18	25.8	0.96
	2	32.1	1.44	36.8	1.58	36.8	1.30	28.7	1.00
Ascorbate	1	16.0	0.79	17.0	0.38	19.2	0.50	20.5	0.05
	2	28.4	0.75	27.0	0.66	30.0	0.70	33.7	0.52

Table 4

Uncoupling of oxidative phosphorylation by basic proteins and the effect of preincubation

The mean uncoupling effect of protein is expressed as percentage, calculated from the data presented in Tables 1-3.

Substrate (15 mM)	Non-preincubated mitochondria			Mitochondria preincubated with		
	Incubation with					
	protamine	MBP	ribo-nuclease	protamine	MBP	ribo-nuclease
Malate+pyruvate	22	28	6	32	53	11
Succinate	27	9	2	30	67	20
Ascorbate	43	70	32	64	89	57

to 89%. The examined basic proteins inhibited to a greater extent the third phosphorylation coupled to the oxidation of ascorbate, both in non-preincubated and preincubated mitochondria.

Table 5

Effect of preincubation medium on the uncoupling effect of mitochondrial basic protein

Conditions as for Table 1 except that preincubation was carried out also in the ionic medium used for the measurement of oxidative phosphorylations. As substrate, succinate was used.

Mitochondria	Expt. no.	Control		Preincubation with MBP		Incubation with MBP	
		Oxygen uptake	P/O	Oxygen uptake	P/O	Oxygen uptake	P/O
Non-preincubated	1	47.9	1.61			37.6	1.21
	2	44.2	1.53			34.9	1.23
Preincubated in 0.25 M-sucrose - 1 mM-EDTA	1	60.2	1.58	18.6	0.42		
	2	53.5	1.50	30.1	0.74		
Preincubated in ionic medium	1	58.2	1.49	38.3	1.14		
	2	53.4	1.50	38.1	1.02		

Table 5 presents the results of the experiments carried out on mitochondria preincubated with MBP in two media: 0.25 M-sucrose - 1 mM-EDTA, and in the medium used for measurement of oxidative phosphorylations which contained, in addition to sucrose and EDTA, also tris and phosphate buffers, $MgSO_4$, ATP and glucose. It appears that MBP uncoupled the oxidative phosphorylation in the mitochondria preincubated in the ionic medium to the same extent as on direct incubation of fresh mitochondria. Ions present in the medium probably prevented binding of MBP to mitochondria and in this way diminished its uncoupling effect [4].

DISCUSSION

It has been demonstrated that mitochondrial basic protein uncouples oxidative phosphorylations similarly as protamine and ribonuclease. These proteins, excepting ribonuclease, inhibited also electron transport. Their effect was markedly enhanced when mitochondria were preincubated with protein in sucrose solution. On the other hand, when the mitochondria were preincubated with basic protein in a medium containing ions (medium used for measurement of oxidative phosphorylations) the enhancing effect did not appear. This is in agreement with the report of Rivenbark & Hanson [9] that addition of 0.08 M-KCl completely reverses the uncoupling effect of protamine on preincubated mitochondria. Hillar & Rzeczycki [4] have postulated that mitochondria can bind MBP by electrostatic forces and that this binding can be overcome by high concentrations of ions or by macroanions (ganglioside).

The presented results seem to support the suggestion of Hanson [2] that basic proteins reduce the negative sites in mitochondria, needed in binding the phosphorylating enzyme. However, this suggestion does not explain why MBP, which is a less basic protein (30% of basic amino acids), uncouples oxidative phosphorylation to a greater extent than protamine which is a more basic protein (90% of basic amino acids). It may be supposed that the structure and amino acid sequence of MBP are such that it is better adapted to inhibit the mitochondrial systems. It is very probable that mitochondrial basic protein is bound electrostatically to the surface of mitochondria regulating their metabolic processes.

SUMMARY

The uncoupling effect of protamine, mitochondrial basic protein and ribonuclease, on oxidative phosphorylations in rat liver mitochondria was studied. The effect was increased when the mitochondria were preincubated with basic protein in non-ionic 0.25 M-sucrose medium.

Mitochondrial basic protein had the strongest effect, and inhibited oxidative phosphorylations coupled to the oxidation of malate with pyruvate, succinate, and ascorbate.

REFERENCES

[1] Dixon M., *Manometric Methods*, p. 9. University Press, Cambridge 1943.
[2] Hanson J. B. - *J. Biol. Chem.* **234**, 1303, 1959.
[3] Hillar M. & Rzeczycki W. - *Acta Biochim. Polon.* **12**, 133, 1965.
[4] Hillar M. & Rzeczycki W. - *Biochim. Biophys. Acta* **97**, 144, 1965.
[5] Layne E., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 3, p. 450. Academic Press, New York 1957.
[6] McDonald M., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 1, p. 270. Academic Press, New York 1955.
[7] McEwen B. S., Allfrey V. G. & Mirsky A. E. - *J. Biol. Chem.* **238**, 758, 1963.

[8] Reiner E. (ed.), *Standard Methods of Clinical Chemistry*, vol. 1, p. 84. Academic Press, New York 1953.
[9] Rivenbark W. L. & Hanson J. B. - *Biochem. Biophys. Res. Commun.* **7**, 318, 1963.
[10] Rzeczycki W. - *Acta Biochim. Polon.* **10**, 279, 1963.
[11] Rzeczycki W., Grudzińska A. & Hillar M. - *Acta Biochim. Polon.* **9**, 295, 1962.
[12] Rzeczycki W., Grudzińska A., Hillar M. & Wszelaki-Lass E. - *Acta Biochim. Polon.* **10**, 49, 1963.

ROZKOJARZENIE OKSYDACYJNYCH FOSFORYLACJI PRZEZ BIAŁKA ZASADOWE

Streszczenie

Zbadano wpływ protaminy, mitochondrialnego białka zasadowego oraz rybonukleazy na oksydacyjne fosforylacje w mitochondriach wątroby szczura. Efekt rozkojarzający znacznie wzrastał, gdy mitochondria preinkubowano z białkiem zasadowym w środowisku niejonowym — 0.25 M-sacharozie.

Mitochondrialne białko zasadowe wywierało najsilniejszy wpływ i rozkojarzało oksydacyjne fosforylacje sprzężone z utlenianiem jabłczanu z pirogronianem, bursztynianu oraz askorbinianu.

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THE REDUCTION OF UBIQUINONE IN RAT LIVER MITOCHONDRIA ASSOCIATED WITH THE OXIDATION OF CHOLINE

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Our earlier results [14] indicated that ubiquinone (UQ) can be reduced by glycero-1-phosphate dehydrogenase. Since this enzyme is an iron-containing flavoprotein like the succinate and $NADH_2$ dehydrogenases, we have suggested that ubiquinone may act as an electron acceptor toward many iron-flavoprotein enzymes forming a link between these enzymes and the cytochrome system.

The present paper reports some data on the reduction of ubiquinone by choline dehydrogenase, an iron-flavoprotein enzyme, as well as betaine aldehyde dehydrogenase localized in mitochondria, which was found to oxidize betaine aldehyde, at variance with the results of other authors, without the participation of NAD.

First experiments dealing with this problem have been presented in 1963 at the 1st Polish Congress of Biochemistry in Łódź and published later [13].

MATERIALS AND METHODS

The rats used were of Wistar strain and weighed 200 - 300 g. Rat liver mitochondria were prepared in 0.25 M-sucrose by the procedure of differential centrifugation developed by Schneider & Hogeboom [12]. After one washing the mitochondria were usually suspended to contain about 25 mg. of protein/ml.

Mitochondria depleted of NAD were prepared according to the method of Ernster & Navazio [5].

Soluble choline dehydrogenase was prepared according to Rendina & Singer [11] using *Bathrops atrox* venom instead of that of *Naja naja*. The incubation with the venom was carried out at 30° without choline as protective factor. The supernatant fraction after centrifugation at 105 000 *g* (step IV) was used as the preparation of soluble choline dehydrogenase.

Mitochondrial protein was determined by the biuret method as described by Szarkowska & Klingenberg [15] and calculated from the formula: $17.5 \times \times \Delta E_{546\ m\mu}\ cm.^{-1}$ for the volume of 5 ml. [7].

In the soluble enzyme preparation the content of protein was determined by the method of Lowry *et al.* [8].

Oxidized ubiquinone was determined according to the method of Pumphrey & Redfearn [9] except that the deproteinization with methanol was carried out without pirogallol [4] at room temperature. Oxidized ubiquinone was calculated from the decrease in extinction at 275 - 295 mμ before and after the addition of KBH_4 (ε, 12.25×10^{-3}). The amount found in the blank sample was taken as the total ubiquinone present in mitochondria.

In experiments with added ubiquinone (UQ_6) its redox state was determined as described previously [14] after extraction with cyclohexane. The extract was diluted by adding the same amount of anhydrous ethanol and the content of oxidized ubiquinone was determined by the decrease in extinction at 272 mμ after reduction with KBH_4. ε_{mM}, 13.57 $cm.^{-1}$ was used.

The reduction of cytochrome *c* and 2,6-dichlorophenolindophenol was measured in a recording Eppendorf photometer. The activity of cytochrome *c* reductase was calculated using the millimolar extinction coefficient $\varepsilon_{546m\mu}$, 9.8 $cm.^{-1}$. For 2,6-dichlorophenolindophenol reductase the millimolar coefficient $\varepsilon_{578m\mu}$, 16.1 $cm.^{-1}$ was used. The rate of the reduction of 2,6-dichlorophenolindophenol is dependent to a great extent upon the dye concentration. This dependence is of such a kind that it is impossible to extrapolate the velocity of the reaction to the infinite dye concentration [11]. For this reason the activity (Table 5) was measured at the highest concentration of the dye which was feasible under the experimental conditions.

The following reagents were commercial products: choline chloride and potassium borohydride (Light Co. Ltd., England), NAD (Sigma, U.S.A.), coenzyme Q_6 (Farmochimica Cutolo-Calosi S.p.a., Napoli), semicarbazide, DL-glyceraldehyde, malate, acetaldehyde, propionaldehyde were of Polish origin. Triton X-100 was kindly given by Rohm & Haas (Philadelphia, Pa., U.S.A.).

Betaine aldehyde (formylmethyltrimethyl ammonium ion) was synthesized according to Bergel *et al.* [1]. The product was dissolved in a minimum quantity of glacial acetic acid, 7 volumes of acetic anhydride were added and the mixture stored in the cold. The crystals of betaine aldehyde chloride were dried over phosphorus pentoxide under reduced pressure.

RESULTS

It is known that in freshly prepared rat liver mitochondria under influence of KCN ubiquinone is maintained in the reduced form by the endogenous substrates. In our experiments the addition of ADP and prolonged aeration (30 min.) in the medium described by Redfearn & Pumphrey [10] or Bianchi & Azzone [2] did not induce the oxidation of ubiquinone because probably the rate of oxidation of the endogenous substrates was too low even in the presence of ADP to exhaust them entirely. On the other hand amytal (3 mM), arsenite (2 mM), or malonate (4 mM) added to block the electron flow from endogenous substrates *via* NAD, and from succinate, did not change appreciably the reduction state of ubiquinone, although under those conditions the mitochondrial NAD became oxidized. It seems

possible that the reduction of ubiquinone is a consequence of the metabolism of fatty acids whose oxidation in liver mitochondria, in contrast to other tissues, does not involve the participation of the intermediates of the tricarboxylic acid cycle [3]. Therefore in liver mitochondria malonate had no effect on the redox state of ubiquinone. Thus the remaining even small amount of endogenous substrates either in the presence of KCN or under anaerobic conditions, was sufficiently high to reduce ubiquinone to a considerable extent. This was a serious obstacle in measuring the redox state of ubiquinone in fresh rat liver mitochondria.

Another great difficulty in estimating the reduction of ubiquinone in carefully prepared mitochondria arose from the existence of a strong permeability barrier for choline. It is known that choline enters easily the mitochondria only after freezing and thawing or after treatments resulting in disintegration of mitochondrial membrane. Therefore only mitochondria aged by a single freezing and thawing were used for studying the reduction of ubiquinone.

Reduction of cytochrome c

Rat liver mitochondria oxidized choline to betaine through an intermediate, betaine aldehyde [6]. When choline was added to the aged mitochondria (Table 1), cytochrome *c* was reduced at a conspicuous rate and there was no increase in velocity after the addition of NAD. NAD, however, increased the rate of reaction when betaine aldehyde was used as substrate.

Table 1

Reduction of cytochrome c *by choline, betaine aldehyde and propionaldehyde*

The reaction mixture contained: 50 mM-phosphate buffer, pH 7.6; 41 μM-cytochrome *c*; 1 mM-KCN; 2 mM-substrates; and where indicated 0.5 mM-NAD. Aged mitochondria 0.14 - 0.57 mg. protein/ml. The reduction of cytochrome *c* was measured at room temperature during the first 2 minutes.

Expt. no.	Substrate	Reduced cytochrome *c* (mμmoles/mg. protein/min.)	
		without NAD	with NAD
1	Choline	50.5	50.0
	Betaine aldehyde	10.6	20.4
	Propionaldehyde	0.0	4.3
2	Choline	58.0	57.0
	Betaine aldehyde	8.5	19.4
	Propionaldehyde	0.0	3.9

In NAD-depleted mitochondria (Table 2) the addition of NAD had no effect on the reduction of cytochrome *c* by choline and betaine aldehyde. DL-Glyceraldehyde and malate, the NAD-linked substrates, reduced cytochrome *c* only after

Table 2

Reduction of cytochrome c *by NAD-depleted mitochondria with choline and aldehydes*

Conditions as in Table 1.

Substrate	Reduced cytochrome *c* (mμmoles/mg. protein/min.)	
	without NAD	with NAD
Choline	88.	89.8
Betaine aldehyde	9.7	9.8
DL-Glyceraldehyde	0.0	15.2
Malate	0.0	96.5

addition of NAD. Thus it appears that oxidation of betaine aldehyde in NAD--depleted mitochondria is catalysed by an aldehyde dehydrogenase which does not have a specific requirement for NAD.

Reduction of endogenous ubiquinone

The addition of choline under anaerobic conditions to the aged mitochondria of rat liver resulted in the reduction of endogenous ubiquinone (Table 3). Similar reduction of ubiquinone took place when betaine aldehyde was added as substrate.

The question thus arose whether reduction of ubiquinone was caused by the oxidation of either choline or betaine aldehyde or of both those substrates together.

Table 3

Reduction of endogenous ubiquinone by choline and betaine aldehyde in rat liver mitochondria

The reaction mixture contained: 0.160 M-sucrose, 10 mM-triethanolamine hydrochloride, pH 7.6, and where indicated 5 mM-choline, 5 mM-betaine aldehyde, 10 mM-acetaldehyde, 50 mM-semicarbazide. Aged mitochondria, 4.8 mg. protein/ml. Final volume 2.5 ml. The reaction mixture was aerated for 5 min. at room temp., then placed in a bath at 37° and continuously flushed with N_2. After 3 min. for temperature equilibration, the reaction was initiated by the addition of substrate and the incubation was continued for the next 5 min. under N_2. The reaction was stopped by addition of methanol.

Additions	Oxidized UQ (mμmoles/mg. protein)	Reduction (%)
None	2.9	—
Choline	1.2	59
Betaine aldehyde	1.6	45
Choline+semicarbazide	1.3	55
Betaine aldehyde+semicarbazide	2.8	3
Choline+acetaldehyde	1.2	59
Betaine aldehyde+acetaldehyde	2.7	3

The influence of betaine aldehyde on the reduction of mitochondrial ubiquinone by choline could be excluded by using semicarbazide, the known carbonyl-group binding reagent.

As it is shown in Table 3, semicarbazide did not inhibit the reduction of endogenous ubiquinone by choline but it inhibited to a large extent its reduction by betaine aldehyde. Similar results were obtained with acetaldehyde which is a potent inhibitor of betaine aldehyde dehydrogenase, but which had no effect on the reduction of ubiquinone by choline.

Table 4

Reduction of endogenous ubiquinone by choline and betaine aldehyde in NAD-depleted rat liver mitochondria

Medium and substrate concentration as described in Table 3. Mitochondria, 5.4 mg. of protein/ml.

Additions	Oxidized UQ (mμmoles/mg. protein)	Reduction (%)
None	3.8	—
Choline	1.6	58
Choline+semicarbazide	1.7	55
Betaine aldehyde	2.4	37
Betaine aldehyde+semicarbazide	3.7	3
Acetaldehyde	3.8	0
Propionaldehyde	3.8	0

Since it has been demonstrated by Glenn & Vanko [6] that mitochondrial betaine aldehyde dehydrogenase has a specific requirement for NAD, in NAD-depleted mitochondria the reduction of ubiquinone should not take place after the addition of this substrate. However, the results presented in Table 4 show that both choline and betaine aldehyde reduced ubiquinone in NAD-depleted mitochondria. The reduction by betaine aldehyde was inhibited by semicarbazide similarly as in undepleted mitochondria. On the other hand, acetaldehyde and propionaldehyde had no influence on the redox state of ubiquinone.

Reduction of exogenous ubiquinone

Exogenous ubiquinone (UQ_6) was reduced by choline in the presence of aged rat liver mitochondria. This reduction took place after addition of KCN in a medium containing Triton X-100. The time-course of the reaction is presented in Fig. 1 and, as shown in Fig. 2, the reduction was dependent upon Triton X-100 concentration.

The soluble preparation of choline dehydrogenase reduced ubiquinone under similar conditions. This reduction was also dependent upon the concentration of Triton X-100 (Fig. 2.)

The soluble preparation of choline dehydrogenase was unable to reduce cytochrome *c*, but it reduced 2,6-dichlorophenolindophenol with choline and betaine

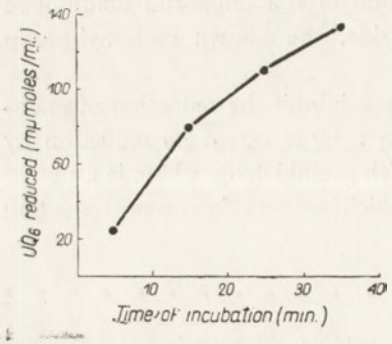

Fig. 1

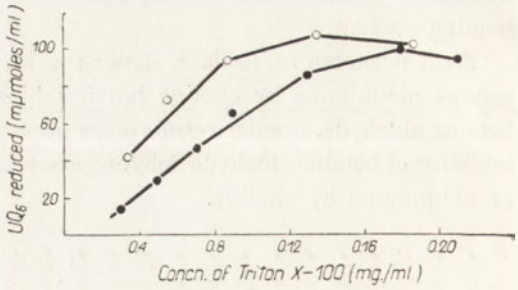

Fig. 2

Fig. 1. Time-course of ubiquinone (UQ_6) reduction by choline in the presence of rat liver mitochondria. Conditions: 10 μmoles of phosphate buffer, pH 7.6, 1 μmole of KCN, 210 mμmoles of UQ_6, 1.5 mg. of Triton X-100, 5 μmoles of choline chloride, 3.9 mg. of mitochondrial protein. Total volume 1 ml.; pH of the reaction mixture 7.6. Incubation was carried out at 37°.

Fig. 2. The effect of Triton X-100 concentration on the reduction of ubiquinone by choline. Conditions: 8 μmoles of phosphate buffer, pH 7.6, 1 μmole of KCN, 192 mμmoles of UQ_6, 5 μmoles of choline chloride, 0.485 mg. of protein of soluble choline dehydrogenase preparation or 4.35 mg. of protein of rat liver mitochondria. Total volume 1 ml.; pH of the reaction mixture 7.6; incubation 15 min. at 37°. (●), Mitochondria; (○), soluble choline dehydrogenase.

aldehyde as substrates (Table 5). The addition of NAD had no effect on this reaction with either substrate. Propionic aldehyde and DL-glyceraldehyde were not oxidized and the addition of NAD had no effect. The reduction of ubiquinone in the presence of the soluble enzyme preparation was equally fast with choline or betaine aldehyde as substrate (Table 6). Semicarbazide inhibited the reduction of ubiquinone by betaine aldehyde, but concentrations required for complete inhibition were four times higher than those needed for the reduction of endogenous ubiquinone in mitochondria.

Table 5

Reduction of 2,6-dichlorophenolindophenol with choline and aldehydes by soluble choline dehydrogenase

The reaction mixture contained: 50 mM-phosphate buffer, pH 7.6, 2 mM-substrates, 40 μM-NAD 0.1 mM-2,6-dichlorophenolindophenol, 1 mM-KCN, 0.06 mg./ml. of enzyme protein

Additions	Reduced 2,6-dichlorophenolindophenol (mμmoles/mg. protein/min.)
Choline	34
Choline+NAD	34
Betaine aldehyde	19
Betaine aldehyde+NAD	19
DL-Glyceraldehyde	0
Propionaldehyde	0
Propionaldehyde+NAD	0

DISCUSSION

The obtained results demonstrate that in rat liver mitochondria endogenous ubiquinone is reduced by choline. Also exogenous ubiquinone can be reduced by choline in the presence of mitochondria or a soluble choline dehydrogenase. A lack of inhibition of this reduction by semicarbazide, the known aldehyde-binding compound, excludes the possibility of ubiquinone reduction by betaine aldehyde, the first product of choline oxidation. This is supported by the fact that acetic aldehyde which inhibits betaine aldehyde dehydrogenase does not affect the reduction of ubiquinone by choline.

Table 6

Reduction of ubiquinone (UQ_6) by soluble choline dehydrogenase

Reaction mixture contained: 8.5 μmoles of phosphate buffer, 1.2 μmoles of KCN, 1.2 mg. of Triton X-100, 145 mμmoles of UQ_6, and where indicated 0.6 μmoles of NAD. pH of the mixture 7.6. Total volume was 1 ml. Enzyme protein 0.485 mg./ml. Incubation 10 min. at 37°.

Additions	Reduced UQ_6 (mμmoles)
None	0.0
Choline	70.0
Choline+semicarbazide, 100 mM	69.5
Betaine aldehyde	66.0
Betaine aldehyde+semicarbazide, 50 mM	44.0
Betaine aldehyde+semicarbazide, 100 mM	22.0
Betaine aldehyde+semicarbazide, 200 mM	0.0
Choline+NAD	70.1

The amounts of betaine aldehyde which are formed during choline oxidation within 5 min. seem to be too low to influence the degree of ubiquinone reduction. However, if betaine aldehyde was added to the liver mitochondria in substrate amount, ubiquinone was reduced. As this reaction took place also in NAD-depleted mitochondria and was not stimulated by the addition of NAD, it seems that the enzyme catalysing this reaction is not NAD-dependent. Such an enzyme has not been described so far. At variance with the known NAD-dependent betaine aldehyde dehydrogenase from mitochondria, this enzyme did not oxidize acetic, propionic and DL-glyceric aldehydes but was specific for betaine aldehyde.

Choline dehydrogenase is the fourth iron-containing flavoprotein enzyme which can reduce ubiquinone like $NADH_2$, succinate and glycero-1-phosphate dehydrogenases do. This is in agreement with the suggestion [14] that ubiquinone is an acceptor for the flavoprotein enzymes containing iron, and that it forms a diffusible link between many flavoprotein dehydrogenases and the cytochrome system.

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SUMMARY

1. Choline added to liver mitochondria caused the reduction of endogenous ubiquinone. The participation of betaine aldehyde was excluded by semicarbazide.

2. In NAD-depleted mitochondria choline as well as betaine aldehyde reduced the endogenous ubiquinone. This suggested that the latter reaction was catalysed by an enzyme which was not NAD-linked. This enzyme was specific for betaine aldehyde.

3. The exogenous ubiquinone was reduced by choline and betaine aldehyde in the presence of mitochondria and soluble preparation of choline dehydrogenase.

REFERENCES

[1] Bergel F., Cohen A. & Hindley N. C. - *J. Chem. Soc.* (London) 1438, 1950.
[2] Bianchi G. & Azzone G. F. - *J. Biol. Chem.* **239**, 3947, 1964.
[3] Bode Ch. & Klingenberg M. - *Biochem. Z.* **341**, 271, 1965.
[4] Erecińska M. & Szarkowska L. - *Acta Biochim. Polon.* **12**, 291, 1965.
[5] Ernster L. & Navazio F. - *Biochim. Biophys. Acta* **26**, 408, 1956.
[6] Glenn J. L. & Vanko M. - *Arch. Biochem. Biophys.* **82**, 145, 1959.
[7] Goebell H. & Klingenberg M. - *Biochem. Z.* **340**, 441, 1964.
[8] Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. - *J. Biol. Chem.* **193**, 265, 1951.
[9] Pumphrey A. M. & Redfearn E. R. - *Biochem. J.* **76**, 61, 1960.
[10] Redfearn E. R. & Pumphrey A. M. - *Biochem. Biophys. Res. Commun.* **3**, 650, 1962.
[11] Rendina G. & Singer T. P. - *J. Biol. Chem.* **234**, 1605, 1959.
[12] Schneider W. C. & Hogeboom G. H. - *J. Biol. Chem.* **183**, 123, 1950.
[13] Szarkowska L. - *Postępy Biochemii* **10**, 77, 1964.
[14] Szarkowska L. & Drabikowska A. K. - *Life Sciences* No. 7, 519, 1963.
[15] Szarkowska L. & Klingenberg M. - *Biochem. Z.* **338**, 674, 1963.

REDUKCJA UBICHINONU W MITOCHONDRIACH WĄTROBY SZCZURA ZWIĄZANA Z UTLENIANIEM CHOLINY

Streszczenie

1. Cholina dodana do zawiesiny mitochondriów powoduje redukcję endogennego ubichinonu. Możliwość udziału aldehydu betainy w redukcji ubichinonu przez cholinę wykluczono stosując semikarbazyd.

2. W mitochondriach pozbawionych NAD ubichinon redukowany jest zarówno przez cholinę, jak i aldehyd betainy. Wydaje się, że utlenienie aldehydu betainy katalizowane jest przez specyficzny enzym nie wymagający udziału NAD.

3. Cholina i aldehyd betainy redukują egzogenny ubichinon w obecności mitochondriów lub preparatu rozpuszczalnej dehydrogenazy cholinowej.

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SYNTHESIS AND DEGRADATION OF ALKALOIDS IN LUPIN ONTOGENESIS

(LUPINUS ANGUSTIFOLIUS)

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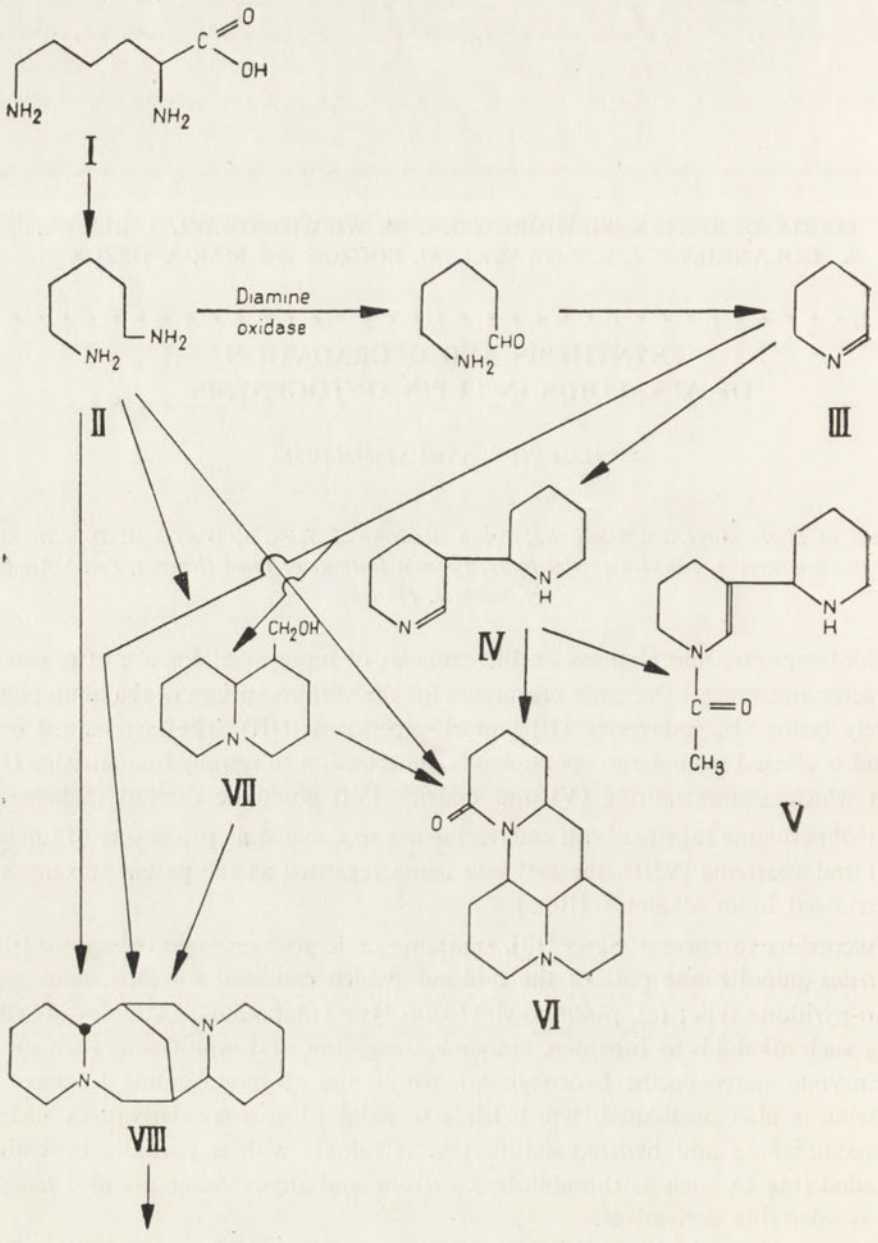
Contemporary speculations on biosynthesis of lupin alkaloids are of a general character and suggest the same precursors for the various species of the lupin plants, namely lysine (I), cadaverine (II) and Δ'-piperideine (III). The last named compound is alleged to undergo spontaneous dimerization to tetrahydroanabasine (IV), from which ammodendrine (V) and matrine (VI) could be derived (Scheme 1).

Δ'-Piperideine together with cadaverine are recognized as precursors of lupinine (VII) and sparteine (VIII), the last one being regarded as the parent substance of all oxidized lupin alkaloids [10, 5].

According to current views [13], sparteine undergoes enzymic oxidation within the *trans*-quinolizidine part of the molecule which can lead to (a), lactam type; (b), α-pyridone type; (c), β-amino-vinyl-keto type compounds, examples of which being such alkaloids as lupanine, aphyline, anagyrine, and multiflorine (Scheme 2).

Enzymic stereospecific hydroxylation within the *cis*-quinolizidine fragment of sparteine is also postulated, which leads to axial 13-hydroxy derivatives such as hydroxylupanine and hydroxymultiflorine. Alkaloids with a partially or entirely degraded ring D, such as rhombifoline, cytisine and angustifoline are also recognized as sparteine derivatives.

Current biosynthetic speculations have not suggested any mechanism of the oxidative and degradative reactions of sparteine and they have not taken into account the dynamism of accumulation of sparteine and its derivatives during the ontogenesis of various species of lupin plants. The hypotheses concerning the pathway of transformations of one alkaloid into another were based first of all on the relative radioactivity measurements of the alkaloid fractions which were isolated from plants previously fed with labelled precursors or grown in the atmosphere of $^{14}CO_2$ [2, 16, 6, 15, 1, 14].



Oxidized lupin alkaloids of the sparteine type

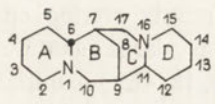
lupanine, anagyrine, hydroxylupanine, multiflorine, cytisine, rhombifoline, angustifoline

Scheme 1. Biosynthesis of lupin alkaloids according to Robinson and Mothes.

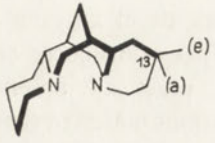
Our investigations on the biosynthesis of lupin alkaloids are at the stage where we are mainly interested in establishing chemical and biochemical links between all known alkaloids in a particular lupin variety.

We propose that alkaloid A may be the precursor of alkaloid B on the basis

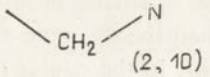
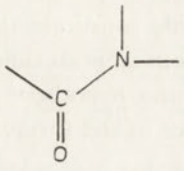
OXIDIZED LUPIN ALKALOIDS OF THE SPARTEINE TYPE

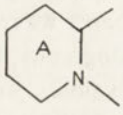
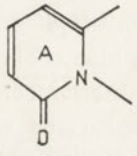


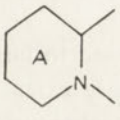
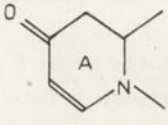
sparteine
VIII



1. OXIDATION OF trans-quinolizidine FRAGMENT OF MOLECULE :

a/   lupanine
aphyline

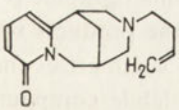
b/   anagyrine

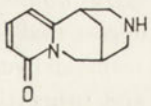
c/   multiflorine

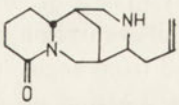
2. HYDROXYLATION OF cis-quinolizidine FRAGMENT OF MOLECULE ONLY AT C_{13}, AND IN AXIAL POSITION :

hydroxylupanine
hydroxymultiflorine

3. VARIOUS PRODUCTS OF DEGRADATION OF cis-quinolizidine FRAGMENT OF MOLECULE :


rhombifoline


cytisine


angustifoline

Scheme 2. Enzymic oxidation of sparteine to "normal" alkaloids.

of the following data: (a), the dynamism of accumulation and disappearance of the various alkaloids during the course of ontogenesis; (b), the fine structure and chemical characteristics of the investigated alkaloids.

We were led to our own biosynthetic speculations owing to the discovery and isolation from *Lupinus angustifolius*, *Lupinus albus* and *Lupinus luteus* of a new group of alkaloids of an ester type, which were entirely overlooked by other investigators. These substances are present in relatively large quantities in younger plants and are practically absent in ripe seeds [7, 19, 18, 4]. Our initial semi-quantitative observations indicated that the drop in the content of ester alkaloids (13-hydroxylupanine being the hydramine component) in *Lupinus angustifolius* coincided with the rise of alkaloids normally present in the seeds, hence our suspicion that the last ones emerge at the exclusion of the first [9]. Moreover, the known mechanism of cyclization of angustifoline to 13-substituted lupanine induced us to believe that the ester alkaloids constitute the connecting link between the three-ring angustifoline and the four-ring alkaloids of the sparteine group.

In order to verify this hypothesis we studied the dynamic changes of accumulation and disappearance of the various alkaloids in the course of ontogenesis of the lupin plants.

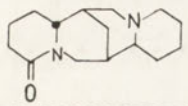
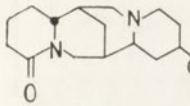
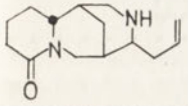
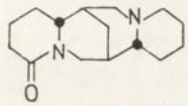
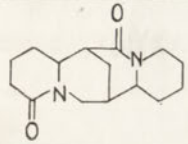
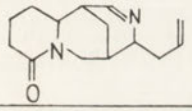
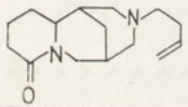
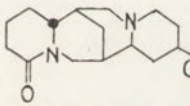
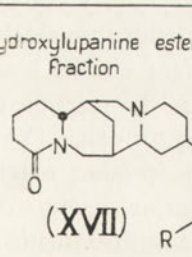
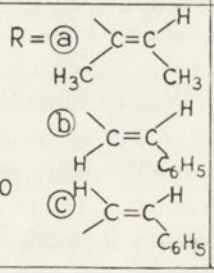
The objects of our investigations were *Lupinus angustifolius*, *Lupinus albus*, *Lupinus luteus* and *Lupinus pilosus*. However, in this paper we report mainly the data concerning *L. angustifolius*. Following paper chromatography, the various alkaloids were quantitatively determined. Simultaneously, alkaloids were isolated in pure state from every investigated ontogenetic phase and the pure compounds were identified by specific rotation, melting point and infrared- and ultraviolet-absorption spectra.

This precaution proved to be essential as it allowed to introduce the necessary correction to the quantitative determinations in those cases where separation by paper chromatography was not very sharp. Our technique permitted to isolate new alkaloids and to determine their specific rotation.

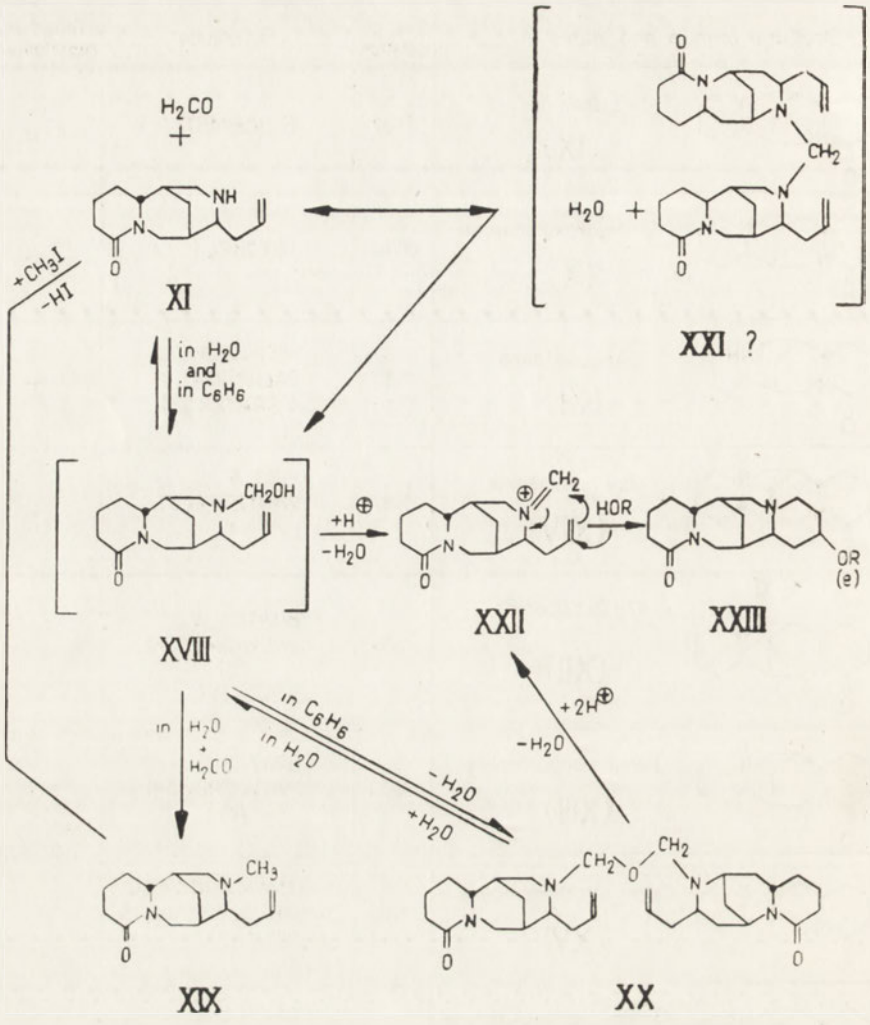
Scheme 3 gives names and formulae of all alkaloids thus far isolated from *L. angustifolius*. In the last column are indicated the alkaloids which were isolated in connection with the present study. As can be seen, four out of eleven alkaloids were not detected in the present work. They all were previously isolated from old mother liquors from which angustifoline had been extracted, and we therefore believe that they were artifacts which arose on prolonged standing of the alkaloid extract.

Furthermore, we succeeded in isolating two new alkaloids. One of these appeared in the second phase of growth of *L. angustifolius* and its chromatographic properties and infrared-absorption spectrum suggest that it is one of the products which can be obtained from angustifoline and formaldehyde. Scheme 4 summarizes the results of our investigation on structures and properties of these labile compounds.

One of these alkaloids appeared in the second phase of growth of *L. angustifolius*, and its chromatographic properties and infrared-absorption spectrum are identical with those of one of the products obtained by condensation of angustifoline (XI) with formaldehyde (Scheme 4).

Structural formula and name	Year of isolation	Reference	Found in reported work
 Lupanine (IX)	1897	E.SCHMIDT (1)	+
 13-Hydroxylupanine (X)	1910	BECKEL (2)	+
 Angustifoline (XI)	1957	WIEWIÓROWSKI GALINOVSKY & BRATEK (3)	+
 α-Isolupanine (XIV)	1957	PIES & WINTERFELDT (4)	+
 17-Oxolupanine (XII)	1959	BRATEK & WIEWIÓROWSKI (5)	
 Dehydroangustifoline (XIII)	1959	BRATEK & WIEWIÓROWSKI (6)	
 Tetrahydrorhombifoline (XV)	1963	BOHLMANN, WINTERFELDT JANIAK, SCHUMANN & LAURENT (7)	
 13-epi-Methoxylupanine (XVI)	1961	BRATEK & WIEWIÓROWSKI (8)	
Hydroxylupanine ester fraction  (XVII) 	1962/63	WIEWIÓROWSKI BRATEK, REIFER (9)	+ + +

Scheme 3. Alkaloids isolated so far from *Lupinus angustifolius*. (1), *Arch. Pharm.* **235**, 192; (2), *Arch. Pharm.* **248**, 451; (3), *Monatsh. Chem.* **88**, 663; (4), *Arch- Pharm.* **290**, 537; (5), *Roczniki Chemii* **33**, 1187; (6), *Roczniki Chemii* **33**, 1187; (7), *Chem. Ber.* **96**, 2254; (8), *Bull. Acad. Polon. Sci., Ser. Sci. Chim.* **10**, 161; (9), *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **10**, 349; *Ser. Sci. Chim.* **11**, 629.



Scheme 4. Condensation products of angustifoline with formaldehyde.

The investigations on the structure and properties of the unstable cyclic compounds formed during the condensation of angustifoline (XI) with formaldehyde are not as yet completed, nevertheless the following conclusions can be drawn: (1), In aqueous medium in presence of excess of formaldehyde the reaction goes through chromatographically detectable *N*-hydroxymethylangustifoline (XVIII) to *N*-methylangustifoline (XIX) identical in all respects with the product obtained in the reaction of angustifoline with methyl iodide. (2), In anhydrous medium there occurs in *statu nascendi* condensation of two molecules of *N*-hydroxymethylangustifoline to the ether of di-*N*-methylangustifoline (XX). This compound contains two *N*-*O*-acetal groups and in aqueous solution is partly hydrolysed to *N*-hydroxymethylangustifoline which is in equilibrium with formaldehyde and angustifoline. Chromatographic analysis of the ether in aqueous medium shows in addition a third

compound which is probably the *N*-*N*-acetal resulting from the condensation of *N*-hydroxymethylangustifoline with angustifoline (XXI). (3), Di-*N*-methylangustifoline ether can be transformed in anhydrous solution into two molecules of an immonium cation (XXII) which in proper medium can undergo the cyclization to the derivatives of lupanine with the equatorial substituent at the 13-position (XXIII).

From all the compounds mentioned in Scheme 4, only *N*-hydroxymethylangustifoline and diangustifoline methane could not be obtained in pure state because of their great lability. Nevertheless, we succeeded in obtaining highly enriched concentrates of these substances, which were confirmed by infrared spectroscopy and chromatographic analysis.

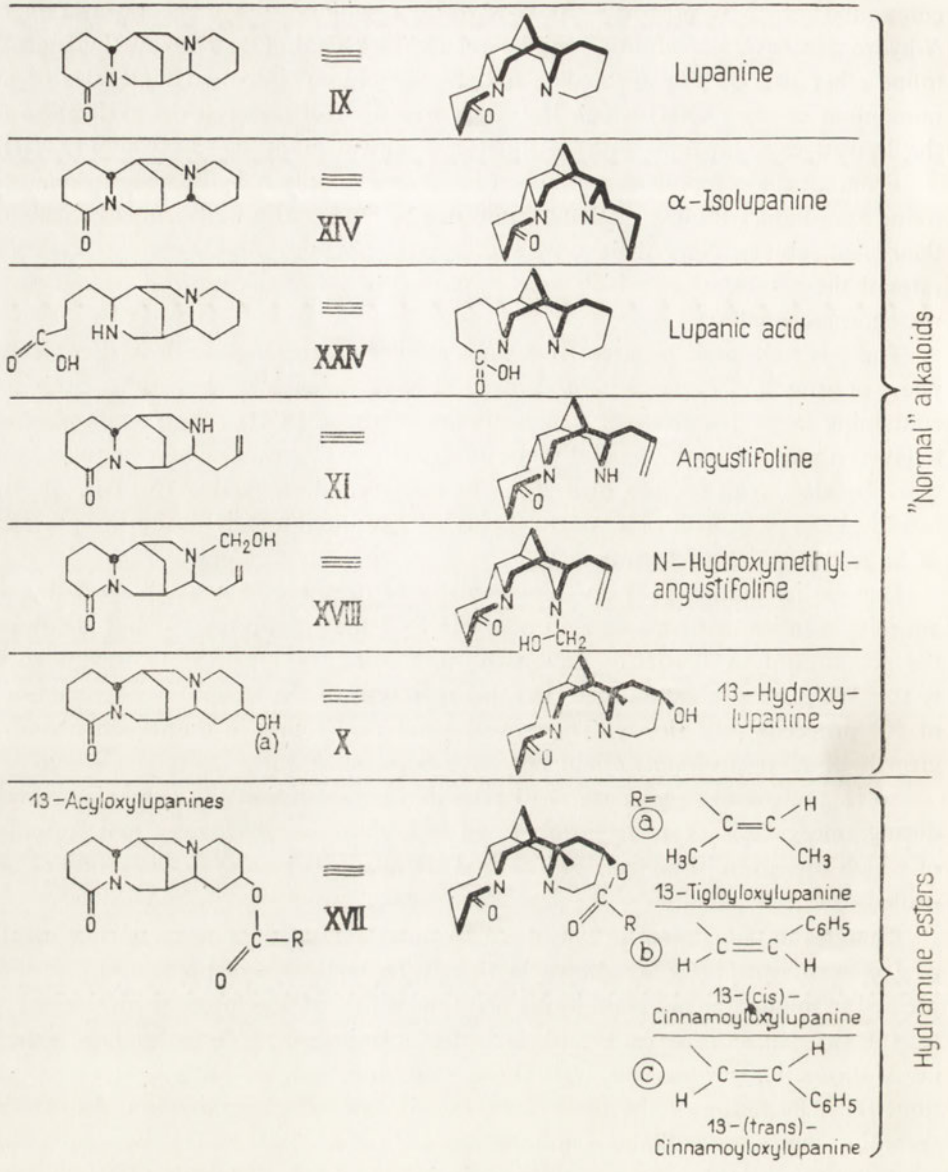
The oily substance isolated from angustifoline mother liquors from the second phase of growth of *L. angustifolius* proved to be very similar to the synthetic mixture containing large quantities of diangustifoline methane (XXI). We therefore tentatively assume that in the second phase of growth of *L. angustifolius*, the plant contains besides angustifoline also its *N*-hydroxymethyl derivative (XVIII). In the fourth phase of growth of *L. angustifolius* we have additionally found the presence of large quantities of lupanic acid.

Our earlier studies [17] on the chemistry of lupanine have established that in aqueous solutions of lupanic acid at a pH 5 - 7 the equilibrium is shifted toward the lactam. But in 1 N-HCl solution the proportion of lupanine to lupanic acid is 1:1. We have also established that the hydrolysis of the lactam at a temperature of 50° proceeds very slowly. Therefore lupanic acid found in the fourth phase of growth of *L. angustifolius* could not have been an artifact.

So in the present paper we shall take into consideration the dynamic changes during ontogenesis of *L. angustifolius* of nine alkaloids the names and formulae of which are given in Scheme 5. The first six alkaloids belong to the group of so-called "normal" alkaloids, the last three to the group of alkaloid esters.

Changes in the content of "normal" alkaloids and alkaloid esters during growth of *Lupinus angustifolius* are shown in Fig. 1. The alkaloids of both groups are expressed in milligrams per plant in the first four phases of development corresponding to: (*I*), two pairs of leaves, i.e. 14 days after germination; (*II*), ten pairs of leaves, i.e. 37 days after germination; (*III*), flower bud formation, i.e. 51 days after germination; (*IV*), flowering of the main stem, i.e. 67 days after germination. As can be seen, the sum of total alkaloids and the sum of "normal" alkaloids grew continually, whereas the alkaloid esters constituting the bulk of total alkaloids up to the second phase of plant growth began to fall markedly at early flowering. This was accompanied by a slowing down in the rise of total alkaloids and an increase in the proportion of "normal" alkaloids.

Figure 2 shows the contents of the individual "normal" alkaloids and the sum of the alkaloid esters during plant growth. Between the phases of two pairs and ten pairs of leaves there was a rapid synthesis of the ester fraction. Its content per plant grew from 7.5 mg. to 165 mg. within 14 days. At the same time hydroxylupanine increased by 38 mg. and lupanine 24 mg. only, together 62 mg. per plant. In the next stage the esters dropped to 154 mg., which is a loss of 11 mg. per



Scheme 5. Structural and steric formulae of alkaloids which were determined quantitatively in the reported work in various phases of ontogenesis of *Lupinus angustifolius*.

plant. At the same time hydroxylupanine grew by 21 mg. and lupanine by 75 mg. per plant, together 96 mg., which is 85 mg. above the ester losses.

In the fourth phase the ester fraction fell by further 52 mg., lupanine and hydroxylupanine rising by the same amount (30 mg. and 22 mg., respectively). In this phase a synthesis of 50 mg. of lupanic acid per plant was noted, hence synthesis of four-ringed alkaloids exceeded even here the rate of degradation of the ester

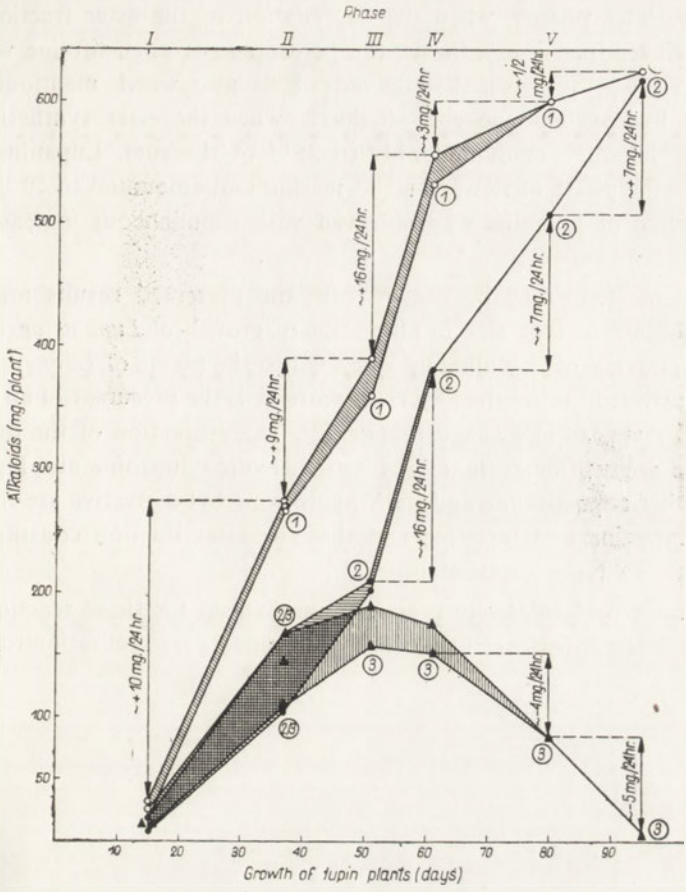


Fig. 1. Content of alkaloids during ontogenesis of *Lupinus angustifolius*. (*1*), Total alkaloids; (*2*), fraction of "normal" alkaloids; (*3*), fraction of hydramine esters.

fraction. The quantities of angustifoline per plant rose similarly, the increases from phase *I* to *IV* being 20, 14.5, and 21 mg., respectively.

It should be pointed out that the highest concentration of *N*-hydroxymethylangustifoline was found in the phase of the greatest intensity of ester synthesis, on the 37th day of growth. α-Isolupanine was the only alkaloid found that did not accumulate in large quantities, the highest concentration being 6 mg. per plant which is a bare 2% of the sum of "normal" alkaloids.

Fig. 3 presents the particular alkaloid content within the sum of the "normal" bases expressed in per cents. Angustifoline rose from 15% to 38% in the phase of two pairs of leaves and constituted the main bulk of total "normal" alkaloids. In the second phase of growth, simultaneously with the most intensive ester synthesis, the content of angustifoline fell to 21%. This decrease, however, was only nominal as at this period the quantity of the *N*-hydroxymethylangustifoline amounted to 14% and so the sum of the two alkaloids amounted to 35% of the "normal" alka-

loids. In the later phases, when the degradation of the ester fraction proceeded at a rate quicker than its synthesis, the percentage of angustifoline was stabilized at about 15 - 20% of "normal" alkaloids. It is also worth mentioning that the quantity of hydroxylupanine rose distinctly when the ester synthesis was at its highest, and lupanine amounted only to 25% of the sum. Lupanine reached its maximum in the phase of flower bud formation and amounted to 50% of the sum. Later a decline of lupanine was observed with simultaneous increase of lupanic acid.

Conclusions that may be drawn from the presented results are as follows. (1), Total absence of sparteine in all phases of growth of *Lupinus angustifolius* and the relative stability of this alkaloid when fed to the plants of *L. angustifolius* allow at least tentatively to reject the idea that sparteine is the precursor of all the alkaloids that normally appear in *L. angustifolius*. (2), The evaluation of the dynamic accumulations of angustifoline, the ester fraction, hydroxylupanine and lupanine seems to indicate that angustifoline and its *N*-hydroxymethyl derivative are the precursors of hydroxylupanine and lupanine and that the ester fraction constitutes the link between the two types of substances.

In Scheme 6 we tentatively present a mechanism for these reactions. Angustifoline through the *N*-hydroxymethyl derivative undergoes cyclization to the unstable

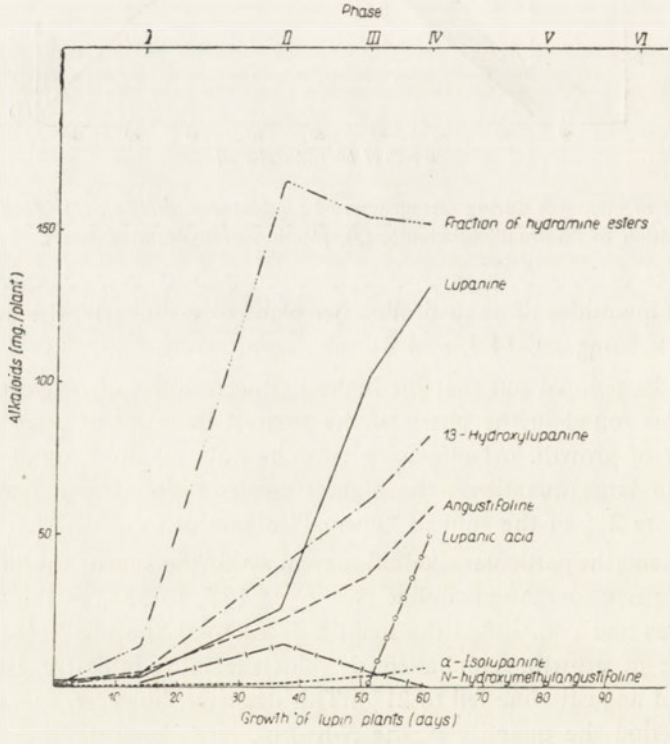


Fig. 2. Content of alkaloids during the first four phases of ontogenesis of *Lupinus angustifolius*.

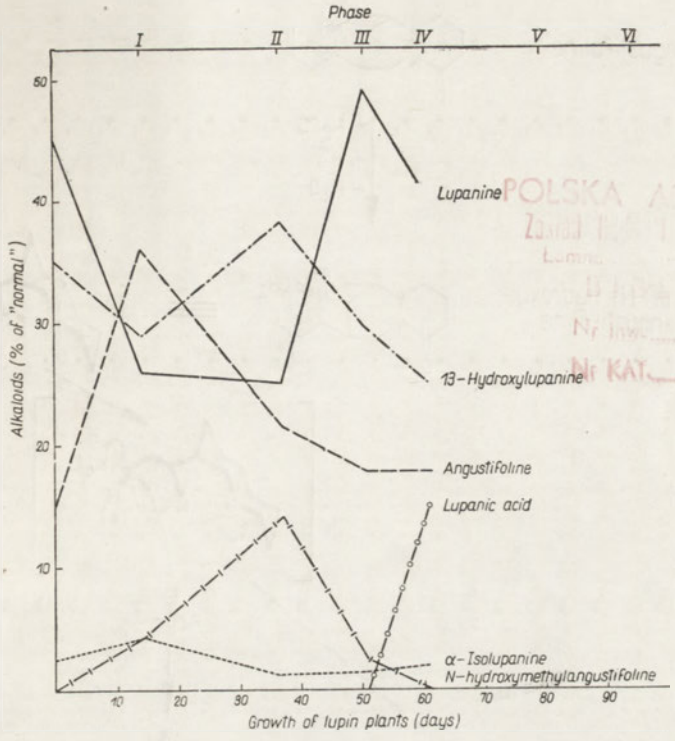
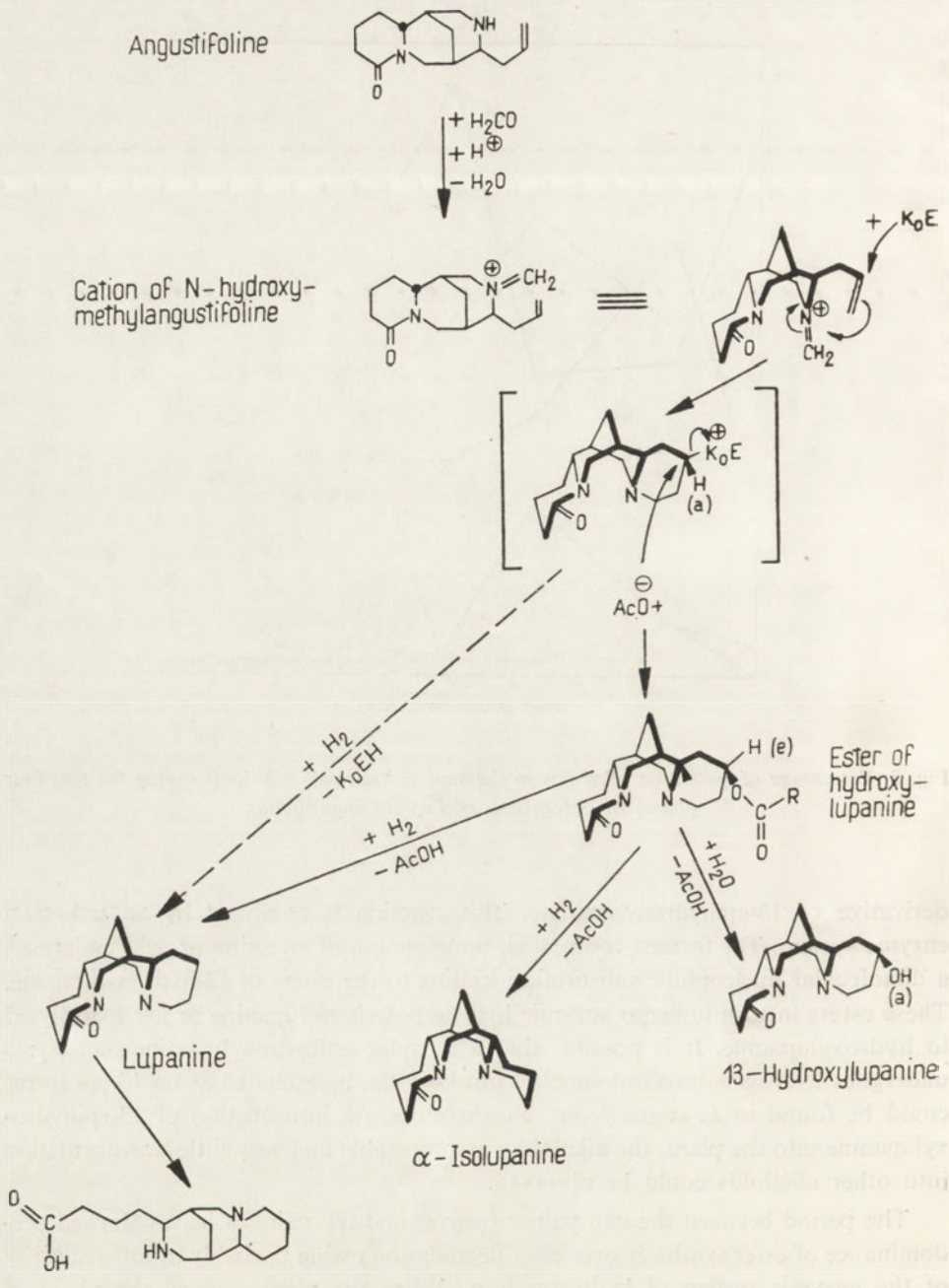


Fig. 3. Percentage of particular alkaloids in the sum of "normal" alkaloids during the first four phases of ontogenesis of *Lupinus angustifolius*.

derivative of 13-epihydroxylupanine. This reaction is catalysed by an unknown enzyme system. The formed compound, under action of an anion or acid undergoes a dimolecular nucleophilic substitution leading to the esters of 13-hydroxylupanine. These esters in turn undergo enzymic hydrogenolysis to lupanine or are hydrolysed to hydroxylupanine. It is possible that a complex epihydroxylupanine - coenzyme undergoes hydrogenolysis but simple hydrolysis can be excluded as no 13-epi forms could be found in *L. angustifolius*. Furthermore, on introduction of 13-epihydroxylupanine into the plant, the alkaloid was very stable and very little transformation into other alkaloids could be observed.

The period between the two pairs of leaves and ten pairs of leaves showed predominance of ester synthesis over ester degradation owing probably to low reactivity of the enzymic system of hydrogenolysis. When the plant reached the phase of flower bud formation hydrogenolysis was observed. The degradation of the esters increased still more in the phase of flowering. At this time lupanine underwent enzymic hydrolysis to lupanic acid, which may well be the connecting link between alkaloids and the general path of nitrogen metabolism in the plant.

At the present stage of our investigation it is not yet possible to place α-isolupa-



Scheme 6. Proposed scheme of alkaloid biosynthesis in *Lupinus angustifolius*.

nine within the biosynthetic chain of the *L. angustifolius* alkaloids. The chemistry of this alkaloid remains largely unknown but our observations tend to suggest that it can undergo oxidative degradation easier than lupanine itself. This, we believe, may be the reason for its small concentration within the plant. It seems

that owing to its considerable chemical reactivity, α-isolupanine together with lupanic acid may constitute the drainage link of alkaloids into the path of nitrogen metabolism. Basing on analogy with the chemistry of 13-hydroxylupanine, we assume that the esters may be also the precursors of α-isolupanine. Enzymic hydrogenolysis leading to lupanine may take place with a change of configuration at C_{11} and then the product of ester degradation would be α-isolupanine (cf. Scheme 6).

Thus far we have not considered the very divergent composition of the ester fraction of the successive phases of plant growth. We believe that this will considerably contribute to the elucidation of the problem studied. The axial-quasi para placement of the tigloyloxyl, *cis*- and *trans*-cinnamoyloxyl substituents leads to compounds of divergent chemical properties with specific adaptability towards hydrolysis and enzymic hydrogenolysis going on directly or with a change in configuration at the nearby C_{11}. Studies on quantitative determination of the various hydramine esters are well advanced.

The interpretations of our results are of course tentative and preliminary. We shall attempt to establish additional facts which will either support our views or compel us to reject our preconceived ideas.

Finally a few informations concerning the alkaloids that appear in *Lupinus albus* and *Lupinus luteus*. Our endeavours to follow the dynamic changes in these two genera of lupinus have encountered considerable difficulties as present knowledge regarding the alkaloid composition of *L. albus* and *L. luteus* is far from being complete. The configuration and structure of some of these compounds is yet to be established. Nevertheless the alkaloid esters isolated by us for the first time are likely to play an important part in the biosynthesis, although their part will certainly not be identical with that of the esters of the *L. angustifolius* series.

Two esters were isolated from the vegetative parts of *L. albus*. The main component was 13-tigloyloxylupanine, accompanied by 13-benzoyloxylupanine. Furthermore from *L. albus* we isolated dehydroalbine which together with the previously obtained *N*-methylalbine may play a similar role in the biosynthesis of multiflorine and hydroxymultiflorine as does angustifoline in the synthesis of lupanine and hydroxylupanine in *L. angustifolius*.

In the early phases of growth of *L. albus*, sparteine was present and as its configuration appeared to be identical with that of the accompanying lupanine a close connection between these two compounds may be assumed. On that evidence we believe that in *L. albus*, apart from the mechanism of synthesis of four-ringed alkaloids through the esters, there may also function the path postulated by Robinson and Mothes namely the synthesis of sparteine from three molecules of lysine or cadaverine, which then undergoes enzymic oxidation to lupanine.

From the vegetative parts of *L. luteus* alkaloids of ester type were also isolated. Chromatographic analysis suggested the presence of four such compounds. Two of these were obtained in a chemically pure form and found to be *cis*- and *trans*-feruloyloxylupinans. Additionally, very labile unsaturated alkaloids were isolated from *L. luteus*. Their properties were very similar to those of the intermediate products

obtained from piperidyllupinan during new synthesis of sparteine by the method of Bohlmann *et al.* [3]. We therefore assume that the ester fraction (acyloxylupinans) in *L. luteus* plays the same part in the biosynthesis of sparteine as does bromlupinan in the chemical synthesis. These data would suggest that the mechanism of alkaloid synthesis in *L. albus* and *L. luteus* is quite different and the studies on the localization of radioactivity in the sparteine molecule after infiltration of [^{14}C]cadaverine should be carefully verified.

EXPERIMENTAL

Plant material

Lupinus angustifolius, high-alkaloid biotype (about 1.5% of alkaloids in the seeds), "Wielkopolski gorzki" was studied. The seeds were sown on April 28th 1964 and for analysis 5 kg. (fresh weight) samples were taken in each of the six phases of ontogenesis. The characteristics of the samples are given in Table 1.

Table 1

Data on material used for analysis

Phase no.	Morphological characteristic of the plant	Weight of 1 plant		The dried plant was	Number and dry weight of samples
		green (g.)	dry (g.)		
I	Two pairs of leaves	13	1.7	without roots	2×20 g. 2×300 g.
II	Ten pairs of leaves	98	18	without roots	4×20 g. 1×300 g. 1×200 g.
III	Flower bud formation	250	42	without roots and the main stems	2×20 g. 2×300 g.
IV	Flowering of the main stem	500	80	without roots and the main stems	2×20 g. 1×300 g.
V	Pod formation	—	—	without roots, stems and the dry sprouts	1×20 g.
VI	Seed ripeness	—	—	without roots, stems and the dry sprouts	1×20 g.

Preliminary experiments showed that dried plants freshly prepared according to the procedure given below have the quantitative and qualitative content of alkaloids very similar to that of the green plant, and therefore dried plants were used for analyses. Since our earlier experiments showed that in the root and lignified parts of the plant there is only about 5% of the total amount of alkaloids, these parts were not analysed and only the weight of the plant to be analysed was establi-

shed. The plants were dried in single layer by a battery of 10 infrared lamps from the distance of about 60 cm., and after 8 hr. they contained only about 10% of water. The finely ground powder was stored in jars.

Preparative methods

For analysis samples of 20 g. or 300 g. of ground dried plants were taken. In the first case the amount of alkaloids and ester fractions was determined. The 300 g. samples were used for isolation of the alkaloids. The procedure of isolation, separation and purification is given below for the 20 g. samples. With few changes this procedure can also be applied for the 300 g. samples.

Extraction of alkaloids, their separation into main fractions and purification

Twenty grams of dried plants were moistened in a mortar with 32 ml. of 20% K_2CO_3, after 20 min. mixed with 16 g. of kieselguhr, placed in a Thiele-Papp extractor and extracted for 3 hr. with 200 ml. of methylene chloride. Then the methylene chloride extract was concentrated by distillation to 10 ml. and poured into a mortar containing 10 g. of cellulose powder. When the 300 g. samples were analysed, the cellulose powder was replaced by 150 g. of kieselguhr but in that case the extraction of alkaloids was more difficult.

After evaporation of methylene chloride the dry cellulose powder was placed in the glass column and the alkaloids were eluted with 50 ml. of 2.5% HCl. The light yellow eluate was twice extracted with ethyl ether, the extract was discarded and the water phase was evaporated to about 15 ml. and then extracted four times with chloroform (A_1). The water phase was neutralized with K_2CO_3, alkalized with 15 ml. of 50% KOH and extracted three times with methylene chloride (B_1). The extracts A and B were dried over Na_2SO_4 and the solvents were evaporated *in vacuo*. The residue A contained the crude ester fraction, and the residue B the crude fraction of "normal" alkaloids. The crude fractions were purified in the following way.

Extract A_1 was evaporated, dissolved in about 5 ml. of 5% HCl and extracted three times with chloroform (A_2). The water phase after alkalization was extracted with methylene chloride (B_2). Then the solutions were dried over Na_2SO_4 and after evaporation of the solvent from A_2 the pure ester fraction was obtained.

The residues B_1 and B_2 were dissolved in 5 ml. of 5% HCl, purified with active charcoal (50 mg.) and filtered through asbestos into a mortar in which the solution was neutralized with K_2CO_3, alkalized with 2 ml. of 50% KOH, mixed with 8 g. of kieselguhr and then placed in a glass column. For the determination of the total alkaloids the elution was carried out with methylene chloride (B_3). For the separation of alkaloids in the 300 g. sample, the kieselguhr with the adsorbed alkaloids was placed in a column containing the same amount of alkalized kieselguhr and the column was washed with petroleum ether (C), ethyl ether (D) and methylene

chloride (E). The eluates were dried by evaporation and the residues contained: B_3, sum of alkaloids; C, lupanine fraction; D, angustifoline fraction; E, hydroxylupanine fraction.

Isolation of pure alkaloids

Lupanine (IX) and α-isolupanine (XIV). Fraction C dissolved in a small amount of methanol was neutralized with perchloric acid. The separated solid after crystallization from methanol had melting point and infrared-absorption spectrum identical with perchlorate of (+)lupanine.

The mother liquor after separation of (+)lupanine perchlorate was evaporated to dryness, alkalized with 20% KOH, mixed with kieselguhr and placed in the column which contained the same amount of alkalized kieselguhr. The alkaloids were eluted with petroleum ether and several fractions were collected. The first fractions consisted of pure α-isolupanine with m.p., $[\alpha]_D$, and infrared-absorption spectrum identical with data for α-isolupanine. The medium fractions contained a mixture of α-isolupanine and lupanine and the last fractions contained pure lupanine only.

Angustifoline (XI) and fraction of N-*hydroxymethylangustifoline (XVIII).* Fraction D was dissolved with heating in 40-fold amount of hexane and the hot solution was filtered through a small column filled with Al_2O_3 (III grade of activity; the ratio of Al_2O_3 to the dissolved substance was 1:2). On cooling, pure angustifoline crystallized from the solution and was identified by melting point and infrared-absorption spectrum. From the concentrated mother liquor two further portions of angustifoline could be obtained.

From some extracts also an oil was obtained with an infrared-absorption spectrum very similar to the spectrum of an old sample of *N*-hydroxymethylangustifoline. Small amounts of substance insoluble in hexane but soluble in ethyl ether, had the same spectrum. Both oils heated for 1 hr. in 1% acetic acid gave a mixture of angustifoline and 13-epihydroxylupanine (XXIII). The two alkaloids were separated in the column with alkalized kieselguhr. Compound XI was eluted with ethyl ether and XXIII with methylene chloride. These data in connection with the infrared-absorption analysis suggest that both oils consist of compounds XI, XVIII, XX and XXI (see Scheme 4).

13-Hydroxylupanine (X). Fraction E was dissolved in a 20-fold volume of acetone, filtered through the column of Al_2O_3 (IV grade of activity; the ratio of Al_2O_3 to the substance was 1:2), and concentrated to 1/3 of the initial volume. After about 12 hr. a substance crystallized out which had a melting point, $[\alpha]_D$, and infrared-absorption spectrum identical with pure (+)-13-hydroxylupanine. From the mother liquor three further portions of X were isolated but compound XXIII could not be detected.

13-Cinnamoyloxylupanine (XVII b and c) and 13-tigloyloxylupanine (XVII a). Fraction A_2 was separated into three chemical individuals on the column of cellulose powder according to the previously published method [4].

Lupanic acid (XXIV). Lupanic acid was not extracted with methylene chloride from the alkalized dried-plant powder. It was isolated by the following procedure. After extraction with methylene chloride of the "normal" alkaloids and the ester fraction, methylene chloride was removed from the material by suction on a water pump and then lupanic acid was eluted with a mixture of *n*-butanol - chloroform (5:1, v/v). To the eluate 3 g. of quartz sand was added and the solvents were removed on a rotary pump *in vacuo*. The residue was washed with ethyl ether and then dissolved in anhydrous *n*-propanol. This operation was repeated and after evaporation of the *n*-propanol, a substance was obtained which chromatographically was identical with lupanic acid. The obtained substance was dissolved in water and neutralized to about pH 6. On heating for 0.5 hr. lupanine was obtained in 90% yield which was confirmed by chromatography as well as by the preparative method described above.

Analytical methods

Quantitative estimation of the particular components of the fraction of "normal" alkaloids and those of the ester fraction was carried out by the nephelometric method according to Reifer *et al.* [8] after chromatographic separation. Usually, fractions A_2 and B_3 obtained from 20 g. of dried plants were taken for analysis. Sometimes analyses were carried out on extracts from 1 g. samples. The applied method did not allow to estimate the amount of α-isolupanine and *N*-hydroxymethylangustifoline because proper chromatographic technique for separation of α-isolupanine and lupanine is not yet known, and *N*-hydroxymethylangustifoline usually undergoes some transformations during chromatography (see Scheme 4). Therefore the amounts of these alkaloids estimated in the extracts obtained during preparative procedure and shown in Figs. 2 and 3 have only approximate value.

SUMMARY

1. The alkaloid composition of *Lupinus angustifolius* was quantitatively investigated in various phases of plant ontogenesis.

2. On the basis of the obtained data a mechanism of alkaloid biosynthesis in *Lupinus angustifolius* was proposed.

REFERENCES

[1] Birecka H. - *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **9**, 341, 1963.
[2] Birecka H. & Ścibor-Marchocka A. - *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **8**, 449, 1960.
[3] Bohlmann F., Winterfeldt E. & Friese U. - *Chem. Ber.* **96**, 2251, 1963.
[4] Bratek-Wiewiórowska M. D., Wiewiórowski M. & Reifer I. - *Bull. Acad. Polon. Sci., Ser. Sci. Chim.* **11**, 629, 1963.
[5] Mothes K. & Schütte R. u.a. - *Angew. Chem. Intern. Ed.* **2**, 341, 1963.
[6] Nowacki E. & Byerrum R. V. - *Biochim. Biophys. Res. Commun.* **7**, 58, 1962.

[7] Reifer I., *II Symposium für Biochemie und Physiologie der Alkaloide, Halle, May 1960.*
[8] Reifer I. & Niziołek S. - *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **7**, 485, 1959.
[9] Reifer I., Wiewiórowski M., Niziołek S., Stawicka D. & Bratek-Wiewiórowska M. D. - *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **10**, 161, 1962.
[10] Robinson R., *The Structural Relations of Natural Products.* Clarendon Press, Oxford 1955.
[11] Schöpf C. - *Angew. Chem.* **69**, 69, 1957.
[12] Schöpf C., Arm H., Benz G. & Krimm H. - *Naturwissenschaften* **38**, 186, 1951.
[13] Schöpf C. & Otte K. - *Chem. Ber.* **89**, 1821, 1956.
[14] Schütte R. & Hindorf H. - *Ztschr. Naturfschg.* **19b**, 855, 1964; Schütte, Hindorf H., Mothes K. & Hübner G. - *Liebigs Ann. Chem.* **680**, 93, 1964.
[15] Schütte H. R., Nowacki E., Kovacs P. & Leibish H. W. - *Arch. Pharm.* **296**, 438, 1963.
[16] Schütte H. R., Nowacki E. & Schäfer Ch. - *Arch. Pharm.* **296**, 20, 1962.
[17] Suszko J., Wiewiórowski M. & Meissner W. - *Roczniki Chemii* **33**, 1015, 1959; *Bull. Acad. Polon. Sci., Ser. Sci. Chim.* **7**, 87, 1959.
[18] Wiewiórowski M. & Bratek M. D. - *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **10**, 349, 1962.
[19] Wiewiórowski M. & Reifer I. - *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **9**, 441, 1961.

SYNTEZA I PRZEMIANY ALKALOIDÓW W ONTOGENEZIE ŁUBINU WĄSKOLISTNEGO

Streszczenie

1. Badano ilościowy skład alkaloidowy *Lupinus angustifolius* w różnych okresach rozwoju rośliny.

2. W oparciu o uzyskane wyniki zaproponowano mechanizm biosyntezy alkaloidów w *Lupinus angustifolius*.

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RECENZJE KSIĄŻEK

J. B. S. Braverman, INTRODUCTION TO THE BIOCHEMISTRY OF FOODS. Elsevier Publishing Co., Amsterdam, London, New York 1963; str. XV+336; cena Dfl. 35, sh. 70, DM 39.

Podręcznik Bravermana przeznaczony jest dla technologów przemysłu spożywczego i zawiera treściwe omówienie chemii i przemian związków naturalnych oraz pewnych ogólnych elementów dynamiki biochemii. W poszczególnych rozdziałach dotyczących węglowodanów, lipidów i białek podano zarówno dane o budowie, własnościach chemicznych i występowaniu, jak również opisano zmiany zachodzące w budowie tych związków pod wpływem czynników enzymatycznych i nieenzymatycznych. W podobny sposób ujęty został rozdział omawiający witaminy. Osobne, specjalne rozdziały poświęcono chlorofilowi, karotenoidom, pektynom, glikozydom antocyjanowym i antoksantynowym oraz olejkom aromatycznym. W każdym z tych rozdziałów zwrócono uwagę na zmiany w strukturze związków naturalnych zachodzące w warunkach przerobu przemysłowego materiałów biologicznych oraz podano środki zapobiegające tym zmianom. Rozdział omawiający koloidy zarówno zaznajamia czytelnika z zagadnieniami tworzenia emulsji, pian i dyspersji związków stałych w produktach przemysłowych, jak również wprowadza w strukturę żywej materii. Dwa rozdziały o enzymach i kinetyce enzymatycznej wprowadzają w przemiany zachodzące w komórce. Spośród procesów biochemicznych obszerniej omówiono jedynie fotosyntezę i fermenta ję. Zagadnienia spalania poruszono w związku z występowaniem w komórkach kwasów organicznych, a z procesów utleniań biologicznych zwrócono uwagę na te, które prowadzą do ciemnienia tkanek roślinnych i przeciwstawiono je omówionym w następnym rozdziale nieenzymatycznym reakcjom brunatnienia opartym na reakcji Maillarda.

Układ podręcznika odbiega niewątpliwie od innych podstawowych podręczników biochemii, w których autorzy zwykle oddzielają omawianie statyki biochemii od przemian, które poprzedza wykład o katalizatorach biologicznych. Powstaje pytanie, czy korzystający z podręcznika student, który na pierwszych kartach książki czyta w związku z omawianiem chlorofilu o cyklu Calvina dotyczącym fotosyntezy, w połowie książki znajduje ogólne informacje o enzymach, a na ostatnich kartach zaznajamia się z ogólnymi mechanizmami utleniania, potrafi sobie wyrobić pogląd o charakterze przemian składających się na zjawiska anabolizmu i katabolizmu w żywych komórkach.

Pewne zastrzeżenia budzi także wybór zagadnień ogólnych. Tak np. omówiono dokładnie równanie Michaelisa a nie podano ogólnych informacji o aktywacji i inhibicji enzymów, podano mechanizm biosyntezy izoprenoidów, a nie omówiono chociażby w ogólnym zarysie syntezy białka. Enzymom niebiałkowym poświęcono specjalny podrozdział, w którym Autor podaje w wątpliwość ogólny białkowy charakter enzymów rozważając budowę aminopeptydazy złożonej z szeregu podjednostek, w skład których wchodzą, obok nielicznych aminokwasów, cukrowce, fosfor i zasady purynowe. Wydaje się, że w podstawowym podręczniku rozważania tego rodzaju mogą doprowadzić studentów do błędnych wniosków. Mimo tych zastrzeżeń należy może jednak zawierzyć prof. Goldblithowi, który w przedmowie do książki bardzo wysoko ocenia wykłady prof. Bravermana na wydziale technologii żywienia w Massachusetts Institute of Technology ora.. stosowaną przez niego metodę dydaktyczną.

W opracowaniu podręcznika oparto się na najważniejszych odkryciach biochemicznych i dyskusjach przeprowadzonych do r. 1960. Z tego pewnie względu nie zostało unowocześnione nazewnictwo enzymów; podana klasyfikacja dzieląca enzymy na dwie grupy: hydrolazy i desmolazy jest całkowicie nieaktualna. Odnośniki do piśmiennictwa obejmują głównie artykuły przeglądowe,

gdyż na tym etapie poznawania przez studentów biochemii związków naturalnych Autor uważa za bardziej celowe pogłębianie wiadomości ogólnych niż poznawanie wycinkowych prac oryginalnych. Zwraca również uwagę niejednokrotny brak precyzji w sformułowaniach np. „ryboza jest ważnym składnikiem enzymów" (str. 55). Owe niedopatrzenia prawdopodobnie wyniknęły z faktu, że prof. Braverman, który zmarł w r. 1962, nie zdążył przeprowadzić do końca korekty swego podręcznika.

Konstancja Raczyńska-Bojanowska

J. E. Falk, PORPHYRINS AND METALLOPORPHYRINS, Elsevier Publishing Co., Amsterdam, London, New York 1964; str. 266; cena Dfl. 37,50, sh. 75, DM 42.

Książka omawia chemię ogólną i analityczną porfiryn i metaloporfiryn w największym skrócie. Przedstawia zagadnienia strukturalne w aspekcie nie tylko klasycznej chemii organicznej, ale również z punktu widzenia fizyko-chemicznego i nowoczesnej teorii kwantowej wiązań. Omówione są również zagadnienia stabilności, własności powierzchniowych, zdolności wiązania makromolekuł porfiryn, a następnie przeniesione są te same rozważania na związki porfiryn z metalami. Szczególnie dokładnie omówiono własności oksydoredukcyjne metaloporfiryn oraz ich własności spektralne łącznie z widmami fluorescencyjnymi w podczerwieni i ich teoretyczną interpretacją. Również omówienie magnetycznego rezonansu jądrowego i rezonansu spinu elektronowego znalazło swe miejsce. Natomiast są potraktowane raczej powierzchownie połączenia porfiryn z białkami.

W drugiej części Autor omawia szczegółowo sprawy metodyczne związane z preparatyką i analityką porfiryn. Czytelnik znajduje w tej części opisy różnorodnych technik, począwszy od oznaczenia punktów topnienia i metod ekstrakcyjnych aż do różnorodnych technik chromatograficznych i metod organicznych, stosowanych w celu scharakteryzowania porfiryn. Opisy te obejmują również prekursory porfiryn, kwas δ-aminolewulinowy i porfobilinogen. W postaci załączników są zamieszczone dane dotyczące widm absorbcyjnych i zależności strukturalnych między poszczególnymi związkami.

Książka, która zawiera 757 pozycji piśmienniczych, jest bardzo cennym wkładem, umożliwiającym szybką orientację w tej bardzo złożonej i tak ważnej dziedzinie biochemii. Od czasów znanej monografii Lemberga i Leggego z roku 1949 nie ma książki, która w tak autorytatywny sposób streszczałaby osiągnięcia ostatnich lat w tej dziedzinie. Autor, uczeń Lemberga i Rimingtona, posiadający poważny własny wkład badawczy, może być uważany za autorytet w zakresie chemii porfiryn.

Sposób, w jaki specjalistyczna wiedza w zakresie chemii porfiryn jest wbudowana w gmach współczesnej chemii i jej podstaw fizykalnych, zwłaszcza teorii wiązań chemicznych, budzi podziw i jest niezbyt często spotykanym zjawiskiem. Lektura książki wymaga dobrego przygotowania i może pobudzać specjalistów chemii porfiryn do uzupełniającej lektury w zakresie podstawowych zagadnień fizyko-chemicznych.

Edward Kowalski

PROGRESS IN THE CHEMISTRY OF FATS AND OTHER LIPIDS (R. T. Holman, ed.) Vol. 7. Pergamon Press, Oxford 1964; str. XI+308; cena 80 s.

Pozycja wydawnicza firmy Pergamon Press pt. Progress in the Chemistry of Fats and Other Lipids stanowi siódmy tom tej serii zapoczątkowanej w 1954 roku pod redakcją Holmana, Lundberga i Malkina. Recenzje dwóch ostatnich tomów, szóstego i siódmego ukazały się ostatnio w polskiej prasie naukowej (P. Włodawer, *Postępy Biochemii* **10**, 416, 1964, D. Hulanicka, *Postępy Biochemii* **11**, 88, 1965 i Z. Kaniuga, *Postępy Biochemii* **11**, 89, 1965). Seria ta stanowi jedną z pierwszych, które zostały całkowicie poświęcone tłuszczom i innym lipidom. Od chwili jej zapoczątkowania rozpoczęto publikację dwóch czasopism z tej samej dziedziny. Są to *Journal of Lipid Research*

i specjalna seria stanowiąca część *Biochimica et Biophysica Acta*, p.t. *Lipids and Lipid Metabolism*. Celem tych ostatnich czasopism jest publikowanie prac oryginalnych, omawiana seria natomiast zawiera artykuły poglądowe. Autorami ich są wybitni znawcy przedmiotu. Poszczególne tomy serii ukazały się w latach 1952, 1954, 1955, 1957, 1958, 1962 i 1964 i zawierają ogółem 56 monograficznych opracowań, z czego połowa została poświęcona chemii i fizykochemii kwasów tłuszczowych, tłuszczów i innych lipidów, czwarta część własnościom biologicznym, enzymologii, resorpcji i znaczeniu lipidów w naukach lekarskich a reszta artykułów ma za przedmiot zagadnienia przemysłowe związane z tłuszczami. Kilka artykułów odnosi się do nowych metod analitycznych. Omawiana seria ma więc szeroko zakrojone ramy. Istotne jej cechy czynią z niej podstawowe źródło uporządkowanych i krytycznie przedstawionych informacji z dziedziny nauki o związkach tłuszczowych.

Tadeusz Korzybski

J. Hadži, THE EVOLUTION OF THE METAZOA. Pergamon Press, Oxford 1964; str. XII+ +500, cena 50 £.

Współczesna systematyka zoologiczna nie ogranicza się do klasyfikowania zwierząt wedle ich cech morfologicznych, ale uwzględnia w równej mierze wyniki badań fizjologicznych, genetycznych i biochemicznych. Idealna systematyka powinna więc przedstawiać zarazem drzewo genealogiczne świata zwierzęcego. Musi zatem żywo interesować biochemika porównawczego, dla którego stanowi źródło inspiracji do podejmowania porównawczych badań, jak i nić przewodnią przy porządkowaniu i interpretacji wyników. Dlatego też uważam za stosowne zasygnalizować na łamach *Acta Biochimica Polonica* ukazanie się dzieła J. Hadži'ego, jakkolwiek merytoryczną ocenę muszę zostawić bardziej kompetentnym specjalistom. Omawiane dzieło przynosi bowiem rewizję całego szeregu pojęć, z którymi zrośliśmy się od czasu, gdyśmy przyjęli ideę ewolucji jako wytyczną w porównawczych badaniach biochemicznych. Przykładowo wymienię tu zarzucenie teorii gastruli, zniesienie typu jamochłonów, zerwanie z koncepcją rozwoju wielokomórkowców w organizmy wielokomórkowe poprzez stadium kolonii, zarzucenie podziału na proto- i deuterostomia, zrezygnowanie z przeciwstawiania coelomata organizmom bez wtórnej jamy ciała itp. Układ proponowany przez Hadži'ego odznacza się prostotą, dzieląc zwierzęta na tylko 4 typy z 42 gromadami. Z ciekawością będziemy śledzić dyskusję nad tym systemem w prasie fachowej.

Józef Heller

III[rd] INTERNATIONAL CONGRESS OF CHEMOTHERAPY. International Society of Chemotherapy. Proceedings (H. P. Kuemmerle & P. Preziosi, eds.) Georg Thieme Verlag, Stuttgart 1964; vol. I, str. 904; vol. II, str. 1848; cena vol. I i II DM 286.

III Międzynarodowy Zjazd Towarzystwa Chemoterapeutycznego odbył się w Sztutgarcie w dn. 22 - 27 lipca 1963 r. Pamiętniki Zjazdu obejmują *in extenso* referaty i komunikaty, zaś dyskusje okrągłego stołu zamieszczono w postaci skróconej.

Rozdział A przedstawia badania nad korelacją między wirusami a rozrostem raka, ze szczególnym uwzględnieniem biologii molekularnej. W tym ujęciu Gouse (Moskwa) wskazuje, że antybiotyk przeciwnowotworowy olivomycin oznacza się korzystnym działaniem klinicznym w leczeniu czerniaka, mięsaka drobnokomórkowego i niektórych postaci raka oskrzeli. Heidelberger (Madison, U.S.A.) omawia biochemiczne aspekty przeciwrakowego działania trójfluorotymidyny i trójfluorodezoksytymidyny w stosunku do gruczolakoraka 755 transplantowanego u myszy. Tu również zamieszczono przeglądowe prace Veldstra (Leyden) o synergizmie leków oraz Grunberga i Price (Nutley, U.S.A.) o mechanizmach powstawania lekooporności.

Rozdział B poświęcono chemoterapii gruźlicy. Omówiono obiektywność i problem etyczny

oceny nowego leku tuberkulostatycznego i bakteriologiczne podstawy leczenia skojarzonego. Krytycznie oceniono połączenia streptomycyny, INH, PAS. Przedyskutowano zagadnienie gruźlicy w zależności od stopnia uprzemysłowienia kraju oraz metody leczenia sanatoryjnego. Wyodrębniono zagadnienie pierwotnej i wtórnej lekooporności, zestawiono wyniki kortykoterapii. Ocenie bakteriologicznej i farmakoterapeutycznej nowego leku tuberkulostatycznego poświęcono 18 komunikatów. Zwracano uwagę na powikłania psychiczne w przebiegu przewlekłego podawania tych leków, a zwłaszcza hydrazynowych inhibitorów oksydaz monoaminowych. Najlepsze wyniki kliniczne uzyskano stosując kanamycynę z cykloseryną i etionamidem.

Rozdział C zawiera 95 komunikatów poświęconych chemoterapii zakażeń bakteryjnych. Interesujące są prace o wpływie antybiotyków na syntezę białek i antymetabolicznym działaniu sulfonamidów. Na uwagę zasługują połączenia niektórych antybiotyków (colimycyna) z sulfonamidami o przedłużonym działaniu oraz współdziałanie penicyliny z tetracyklinami. Zagadnienie oporności na antybiotyki badano *in vitro* i uwzględniając problematykę kliniczną. Wykazano działanie przeciwrobacze, pierwotniakobójcze, cytostatyczne związków nitrofuranowych.

Rozdział D zawiera 24 komunikaty z zakresu chemoterapii chorób wirusowych; miały one na celu ustalenie zależności pomiędzy związkami przeciwwirusowymi czynnymi *in vitro* i *in vivo*, oraz określenie bloku metabolicznego wywołującego takie działanie. Omówiono przeciwwirusowe efekty 2-(α-hydroksybenzylobenzimidazolu) oraz gwanidyny, etioniny, *p*-fluorofenyloalaniny i pochodnych akrydyny. Posługując się wirusem grypy A_2W_{29} inkorporowanym do krwinki czerwonej określono charakterystykę elektrochemiczną niezbędną dla jego absorpcji. Podano nowe metody testowania leków przeciwgrypowych i przeciwparagrypowych. Na zarodku kurczęcia określono działanie przeciw wirusowi grypy 5-nitrofuryloglioksalu i kwasu *p*-(etoksy-β-oksy-5--nitrofurylo)-2-(etyloaminobenzoesowego). Kilka doniesień dotyczyło zapobiegania zakażeniu grypowemu przy pomocy flumidyny.

Tom II obejmuje 149 komunikatów. Rozdział E zawiera prace nad chemoterapią raka. Omówiono mechanizm działania 5-jodourydyny, antagonisty tymidyny, i przedyskutowano możliwość jego klinicznego zastosowania. Określono aktywność przeciwnowotworową następujących związków: cytoksan, DL-sarkolizyna, L-sarkolizyna, chlorambucil, nitromin, mitomycyna C, TEM, Tio-TEPA, OPSPA i myleran. W klinicznej ocenie przeciwnowotworowego działania alkaloidów barwinka za lek z wyboru uznano winblastynę (60% regresji w chorobie Hodgkinsa, 20% w lymphosarcoma). Opisano przeciwrakowe właściwości enomycyny, peptydu pozbawionego działania przeciwbakteryjnego, oraz fleomycyny posiadającej działanie przeciwbakteryjne. Przy użyciu izotopów przebadano losy aktynomycyny w ustroju i mechanizm jej działania. Stwierdzono, że hamuje ona syntezę otoczki białkowej bakteriofagów, zaś mitomycyna C interferuje z metabolizmem RNA komórek prawidłowych i nowotworowo zmienionych.

Napływ informacji o antybiotykach przeciwnowotworowych nie osłabił poszukiwań leku syntetycznego tego typu. Świadczą o tym prace nad alkilowanymi karbominianami oraz wprowadzenie nowej grupy leków cytostatycznych, pochodnych metylohydrazyny. Podobnymi właściwościami oznaczają się estry kwasu alkilosulfonowego. W leczeniu choroby nowotworowej u ludzi względnie zadowalające wyniki uzyskano stosując związki alkilujące (iperyt 1-fenyloalaniny, cyklofosfamid, chlorambucil), hormony sterydowe (testolakton, 17-hydroksyprogesteron), antybiotyki (aktomycyna D, mitomycyna C, streptonigryna, chromomycyna), alkaloidy (winblastyna) oraz inne związki, jak hydroksymocznik, nitrozomocznik, hadacyna, pochodne hydrazyny. W terapii przeciwnowotworowej narządu rodnego zachęcające wyniki uzyskano stosując cyklofosfamid, zaś w leczeniu raka skóry — mitomycynę C i chromomycynę. Najczęstszymi objawami ubocznymi przy stosowaniu leków cytostatycznych są: opóźnione gojenie ran i leukopenia.

W rozdziale F (17 komunikatów) zawarte są dane o nowych penicylinach. Określono właściwości synematyny B i cefalosporyny N o szerszym zakresie działania przeciwbakteryjnego niż benzylopenicylina. Wprowadzono testy naskórne i hemaglutynacyjne umożliwiające wykrywanie potencjalnej alergii na penicylinę. Z *Fusidium coccineum* uzyskano fuzydynę, nowy antybiotyk przeciwgronkowcowy o budowie sterydowej. Omówiono bakterjostatyczne właściwości pochodnych 2,4-dwuaminopirydopirymidyny o własnościach inhibitora reduktazy kwasu dwuhydrofolio-

wego. Wykazano, że kwas nalidyksynowy o budowie nie spotykanej w dotychczasowych grupach leków charakteryzuje się działaniem przeciw bakteriom gram-ujemnym.

Rozdział G omawia leki stosowane w profilaktyce i leczeniu choroby popromiennej. Bardzo skuteczną okazała się marinomycyna wyosobniona ze *Streptomyces marinensis*.

W rozdziale F zawarte są komunikaty na temat terapii chorób tropikalnych. W leczeniu amebiaz zachęcające wyniki uzyskano stosując 2-dwuhydroemetynę. Wytworzono model doświadczalnej amebiazy i chorób wywołanych przez trematody (np. paragonimiasis) i wykazano przydatność preparatu LZ 544 (Hetolin). Określono oporność *Trypanosoma congolense* w stosunku do 4,4-dwuamidynodwuazoaminobenzolu i wykazano przydatność amidynofenoksypirymidyny i pochodnych 8-hydroksychinoliny w zakażeniach wywołanych trypanocydami.

Rozdział I dotyczy terapii chorób z samouczulenia. Powszechne przyjęcie immunologicznej patogenezy małopłytkowości ustaliło pogląd o skuteczności stosowania ACTH i kortykosterydów w tych zespołach. Przebadano wpływ leków cytostatycznych na tworzenie się przeciwciał.

Rozdział S określa przydatność badań farmakokinetycznych w rozwoju chemoterapii. Badania tego typu prowadzą do określenia optymalnej dawki, umożliwiającej uzyskanie odwracalnego efektu bakteriostatycznego. Podano zarys postępowania eksperymentalnego i interpretację kinetyki wchłaniania, rozdziału, metabolizmu i wydalania leków z ustroju.

Ponadto tom II zawiera protokóły 7 dyskusji okrągłego stołu omawiających zagadnienia teratogenności leków chemoterapeutycznych; odpowiedzialności prawno-administracyjnej za dystrybucję tych leków; cele i sposoby działania Międzynarodowego Ośrodka Informacji o Antybiotykach (I.C.I.A.); oraz zagadnienia rozdziału antybiotyków w ustroju. W dyskusji przedstawiono również niektóre zagadnienia chemoterapii i immunologii schorzeń wirusowych, onkolizę doświadczalną wywołaną wstrzyknięciami apatogennych form klostrydialnych, oraz omówiono działanie uboczne leków obniżających krzepnięcie krwi.

Pamiętniki III Międzynarodowego Sympozjum Chemoterapii przedstawiają osiągnięcia z zakresu badań laboratoryjnych i klinicznych nad nowym lekiem etiotropowym, cytostatycznym, tuberkulostatycznym i przeciwwirusowym. Stanowią one zbiór cennych informacji dla biochemika, bakteriologa, farmakologa i klinicysty zainteresowanego tymi zagadnieniami.

Zbigniew Szreniawski

C. C. Brown, G. N. Webb (konsultant), INSTRUMENTATION WITH SEMICONDUCTORS, for Medical Researchers. Charles C. Thomas Publ., Springfield (Ill.) 1964; str. XVII+254; cena 10,50 $.

Ukazanie się tej książki w serii monografii „American Lectures in Objective Psychiatry" stanowi bardzo znamienny wyraz postępu dokonywającego się w ostatnim dziesięcioleciu w stosowaniu najnowszych zdobyczy elektroniki w naukach przyrodniczych w celu podniesienia obiektywności i czułości obserwacji, zwiększenia liczby danych doświadczalnych czy wreszcie ich analizy przy pomocy różnego typu elektronowych maszyn matematycznych. Ten szybki rozwój techniki badawczej zmusza psychofizjologa, neurologa, bionika czy biofizyka do szukania fachowej pomocy inżyniera elektronika przy adaptacji standartowych przyrządów do szczególnych warunków doświadczalnych, przy projektowaniu i budowie unikalnej aparatury pomiarowej, układów analogów itp. Sukces współpracy zależny jest oczywiście od możliwości porozumienia się obu specjalistów, co przy tak różnym ich przygotowaniu fachowym nie jest rzeczą łatwą. Autor, psychofizjolog i elektronik w jednej osobie, postawił sobie w omawianej książce zadanie ułatwienia tego porozumienia poprzez zwięzły wykład teorii i praktyki stosowania półprzewodników w elektronowych układach pomiarowych, dostosowany swym poziomem do możliwości percepcji pracownika naukowego, lekarza lub przyrodnika z wykształcenia. W związku z tym Autor zrezygnował ze ścisłego sformalizowanego opisu przedmiotu oraz z przytaczania szczegółów technologicznych i konstrukcyjnych na rzecz fenomenologicznego opisu, w którym zdecydowany nacisk położony został na wyjaśnienie

charakteru operacji wykonywanych przez poszczególne elementy obwodów elektronicznych oraz ich zespoły. Zachowany został przy tym fachowy język elektroniki.

Na wstępie Autor dyskutuje kryteria właściwego zastosowania przyrządów pomiarowych (rozdz. 1), oraz ogólne założenia ich konstrukcji (rozdz. 2 i 3). Systematyczny wykład poprzedzony jest omówieniem podstawowych pojęć elektroniki i praw rządzących przepływem prądu elektrycznego (rozdz. 4). Rozdział 5 poświęcony jest opisowi najistotniejszych operacji spełnianych przez wyspecjalizowane układy elektroniczne, takich jak wzmacnianie, prostowanie, dzielenie, dodawanie, całkowanie, itp., abstrahując od tego, czy są one oparte na lampach elektronowych czy też elementach półprzewodnikowych. Zgodnie z tytułem książki, w dalszych jej rozdziałach czytelnik spotyka się wyłącznie z podzespołami opartymi na elementach półprzewodnikowych. Ograniczenie to podyktowane jest niemal powszechnym wyparciem przez półprzewodniki lamp elektronowych we współczesnych elektronicznych przyrządach pomiarowych przeznaczonych do obserwacji sygnałów mających swe źródło w żywych organizmach. Podstawowe właściwości półprzewodników stanowiące o ich użyciu zamiast lamp elektronowych omówione są w rozdziale 6.

W ośmiu dalszych rozdziałach Autor daje wyczerpujący przegląd współczesnych zastosowań elementów półprzewodnikowych od najbardziej rozpowszechnionych, jak różnego typu układy wzmacniające, generatory drgań, układy sterujące czy zasilacze (rozdz. 7, 9, 10, 13), do mniej rozpowszechnionych lecz rokujących duże nadzieje na przyszłość. Wśród tych ostatnich warto wymienić urządzenia półprzewodnikowe, których przewodnictwo zmienia się pod wpływem różnych działających z zewnątrz czynników jak światło (fotoopory), ciepło (termistory), siła mechaniczna (rozdz. 8); układy normalnie spotykane w matematycznych maszynach cyfrowych, lecz znajdujące również zastosowanie w bardziej rozbudowanych układach pomiarowych, na przykład przy programowaniu obserwacji przy pomocy szeregu różnych sygnałów z tego samego źródła (rozdz. 11); czy wreszcie magnetyczne metody zapisu, którym poświęcony jest rozdz. 12. Przejrzyste, poglądowe i starannie opracowane graficznie schematy i wykresy w poważnym stopniu ułatwiają czytelnikowi przyswojenie trudnego materiału.

W sumie książka stanowi doskonałe, pierwsze chyba tego typu, wprowadzenie do teorii i praktyki elektroniki układów półprzewodnikowych i zgodnie z intencjami Autora niewątpliwie umożliwia czytelnikowi bez przygotowania w tej dziedzinie nawiązania dialogu z inżynierem elektronikiem.

Kazimierz L. Wierzchowski

H. Hayasaka, J. M. Howard, SEPTIC SHOCK. Experimental and Clinical Studies. Charles C. Thomas Publ., Springfield (Ill.) 1964; str. 86; cena 5.00 $.

Pojawienie się septycznego wstrząsu budzi zawsze nie tylko niepokój lecz także jest źródłem jak najpoważniejszego rokowania, szczególnie u chorych leczonych chirurgicznie. Jak podają w swym zestawieniu Altmeister i Cole (1956) nie udało im się uratować żadnego z 93 chorych, u których w przebiegu posocznicy doszło do stanu zapaści.

Kataklizmy żywiołowe i wojny stanowią okazję do występowania tego rodzaju zaburzeń, dlatego wydaje się oczywiste, że właśnie w okresie I i II wojny światowej poświęcono septycznemu wstrząsowi dużo uwagi i rozpoczęto prace mające na celu wyjaśnienie wielu dotychczas niejasnych zagadnień z zakresu jego etiopatogenezy. W wyniku tych badań stwierdzono, że przyczyną wstrząsu mogą być zarówno egzotoksyny i endotoksyny, jak i szereg innych czynników pojawiających się w czasie infekcji. Ponieważ badania na ludziach z natury rzeczy muszą ograniczać się do kontrolowania niektórych zjawisk w trakcie już rozwiniętego zespołu wstrząsowego w przebiegu posocznicy, dlatego też dla pełnego ich prześledzenia należało się uciec do badań doświadczalnych na zwierzętach. W badaniach tych prześledzono: 1) wpływ endotoksyny na układ krążenia, 2) zachowanie się krążenia pod wpływem egzotoksyny i 3) zespół wstrząsu wyzwolony przez infekcję bakteryjną.

Wpływ endotoksyny, produkowanej przez różne gatunki gram-ujemnych drobnoustrojów, przebadano na dużej serii zwierząt doświadczalnych (myszy, szczury, świnki morskie, małpy i psy).

U wszystkich zwierząt stwierdzono wielkie podobieństwo w reakcjach na podane jady, jak również podobieństwo zmian anatomopatologicznych stwierdzanych podczas sekcji. Natomiast gatunkowe różnice występowały w czasie, jaki upływał od chwili podania toksycznej dawki do pojawienia się pierwszych objawów toksycznych, oraz w intensywności reakcji. Kształtowała się ona różnie wśród różnych gatunków zwierząt w zależności od drogi podania toksyny.

Egzotoksyny, produkowane głównie przez bakterie gram-dodatnie, różne w swym specyficznym działaniu, na różnej drodze mogą prowadzić do wystąpienia zespołu wstrząsu. Ze znacznie bardziej skomplikowanym mechanizmem wstrząsu należy się liczyć we wstrząsie septycznym, gdzie zarówno różnorakie drobnoustroje i rozmaite drogi ich wtargnięcia, jak i niejednakowy stan chorych, odgrywają niemałą rolę. W zależności od sytuacji na pierwszy plan wysuwać się mogą odczyny naczyniowo-ruchowe, zmiany w mięśniu serca, hemokoncentracja itp. W związku z różnymi odczynami w stanie wstrząsu Autor omawia najczęściej stosowane metody jego zwalczania.

W omawianej pracy Autorzy próbowali dokonać przeglądu osiągnięć w badaniach nad mechanizmem wstrząsu w różnych stanach infekcyjnych, głównie jednak z punktu widzenia możliwości praktycznego ich wykorzystania w walce z ciężkim, niejednokrotnie nieodwracalnym wstrząsem. Zadanie wykonali bardzo sumiennie, zestawiając najważniejsze pozycje piśmiennictwa z całego świata. Jednak po przeczytaniu tej książeczki trudno wyrobić sobie pogląd na to, co na drodze tak licznych i żmudnych badań osiągnięto, o ile badania naukowe posunęły naprzód naszą znajomość mechanizmów wstrząsowych i tym samym przyczyniły się do ich zwalczania. Autorzy nie podjęli również próby sformułowania jakichś ogólniejszych wniosków zarówno w sprawie patomechanizmu, jak i pewnych odrębności septycznego wstrząsu. Ten zaś brak sprawia, że oceniane dzieło nie daje spodziewanych korzyści.

Strona wydawnicza książki jest bez zarzutu, świadcząc jak najlepiej o wydawcy.

Marian Górski

M. L. Petermann, THE PHYSICAL AND CHEMICAL PROPERTIES OF RIBOSOMES. Elsevier Publ. Co., Amsterdam, London, New York 1964; str. 258; cena Dfl. 27,50, sh. 55, DM 31.

W ciągu ostatnich kilkunastu lat byliśmy świadkami narodzin i niezwykle bujnego rozwoju nowej rozległej gałęzi biochemii, biochemii biosyntezy białka. Rybosomy jako organelle komórkowe, w których zachodzi synteza polipeptydów oraz odbywa się „odczytywanie" informacji genetycznej zawartej w kwasach nukleinowych, są oczywiście jednym z najważniejszych obiektów badawczych tej nauki. Wielka ilość materiału doświadczalnego nagromadzona w ostatnim czasie w piśmiennictwie biochemicznym wymagała uporządkowania, dlatego też ukazanie się monografii Mary Petermann powitane zostało ze zrozumiałym zainteresowaniem.

Jest to książka przeznaczona dla biochemików i biofizyków interesujących się zagadnieniem biosyntezy białek, strukturą i funkcją rybosomów oraz kontrolą genetyczną budowy białek. Jest ona szczególnie cenna dla tych, którzy pracują w tych dziedzinach, gdyż zawiera wiele cennych informacji, wskazówek pratycznych oraz opisów metod.

Rozdział pierwszy i drugi są poświęcone wprowadzeniu oraz ustaleniu terminologii, zawierają także krótki lecz wyczerpujący rys historyczny zagadnienia.

W trzecim rozdziale opisano występowanie rybosomów w różnych organizmach a także pokrótce wewnątrzkomórkową lokalizację tych cząstek. Czwarty rozdział zawiera opisy metod otrzymywania i oczyszczania rybosomów z różnych źródeł. Najczęściej stosowane metody są opisane dostatecznie szczegółowo, tak że nie wymagają sięgania do prac oryginalnych. Większość tych metod poddano krytycznej ocenie, tak że czytelnik może wybrać sposób najbardziej odpowiadający jego potrzebom. Rozdział piąty jest właściwie dalszym ciągiem poprzedniego, gdyż omawia niezwykle ważne i bardzo trudne zagadnienie oceny czystości otrzymanych preparatów rybosomalnych.

Następne dwa rozdziały są poświęcone budowie oraz chemicznym i fizyko-chemicznym właściwościom rybosomów z uwzględnieniem danych otrzymanych przy pomocy najbardziej nowoczesnej

techniki eksperymentalnej, jak na przykład ultrawirowanie, mikroskopia elektronowa i rozproszenie światła.

Rozdział ósmy omawia wiązanie jonów magnezu przez rybosomy oraz dysocjację i asocjację podjednostek rybosomów.

W rozdziale jedenastym znajdujemy dane o najnowszych osiągnięciach biochemii w zrozumieniu funkcji rybosomów. Omówiono w nim mechanizmy przyłączania do rybosomów przenoszącego RNA oraz przyłączenie sztucznych i naturalnych informacyjnych polinukleotydów i tworzenie polisomów.

Każdy z powyższych rozdziałów posiada bardzo krótkie streszczenie omówionych w nim zagadnień, co bardzo ułatwia orientację w dużej ilości materiału faktycznego zawartego w książce.

Największą zaletą tej monografii jest chyba jej zwięzłość. Autorka, która jest wybitną specjalistką w swej dziedzinie, potrafiła z podziwu godnym kunsztem wyłuskać najważniejsze fakty z „powodzi" prac doświadczalnych na temat rybosomów, zalewających ostatnio literaturę biochemiczną, i podać je czytelnikowi w sposób prosty i zrozumiały.

Bogate piśmiennictwo liczące 796 pozycji obejmuje okres do końca 1963 roku, jednakże w końcu książki znajduje się kilkustronicowe „Addendum" zawierające najbardziej aktualne uzupełnienie do wszystkich rozdziałów oraz dodatkowo 186 pozycji piśmiennictwa z okresu do połowy 1964 roku.

Monografia Mary Petermann zainteresuje niewątpliwie nie tylko specjalistów, lecz także szerokie grono biochemików i biofizyków.

Michał Bagdasarian

ENZYMES IN CLINICAL CHEMISTRY (R. Ruyssen and L. Vandendriessche, eds.) Elsevier Publishing Co., Amsterdam, London, New York 1965; str. VIII+152; cena Dfl. 25, sh. 50, DM 28.

Książka jest zbiorem referatów wygłoszonych na Sympozjum Chemii Klinicznej w Ghent w 1964 r. Na treść tego tomu składa się 16 referatów i streszczeń referatów wygłoszonych na tym sympozjum.

Burzliwy rozwój biochemii klinicznej, wprowadzenie nowych metod, oraz adaptacja metod biochemii teoretycznej zmuszają do bardzo pilnego śledzenia bogatej literatury poświęconej tej tematyce. Należy więc przyjąć z uznaniem nie tylko inicjatywę organizowania sympozjów i spotkań ludzi zajmujących się tą tematyką, ale także publikowania materiałów (referatów i streszczeń) z tych spotkań, właśnie w formie książek.

Zakres zagadnień omawianych w tym tomie jest bardzo szeroki, począwszy od najnowszych osiągnięć techniki badawczej w najszerszym tego słowa znaczeniu aż do omówienia niektórych wad w metabolizmie. Cała grupa referatów poświęcona jest izoenzymom; dyskutowano heterogenność enzymów tkankowych oraz schemat rozmieszczenia enzymów w organach, surowicy i tkance.

Książka przeznaczona jest przede wszystkim dla pracujących w biochemii klinicznej i lekarzy praktyków. Dlatego położony jest nacisk na wyjaśnienie podstawowych zasad pracy, kryteriów czystości oraz trudności, jakie mogą wystąpić przy interpretacji wyników. Prawie we wszystkich referatach Autorzy zwracają uwagę na możliwość błędów metodycznych i na sposoby ich uniknięcia. Cały pierwszy rozdział napisany przez B. Entressangles'a i P. Desnuelle'a poświęcony jest metodyce oznaczania kinetyki reakcji enzymatycznych. Autorzy omawiają czynniki mogące mieć wpływ na szybkość reakcji enzymatycznych, a także zwracają uwagę na właściwy dobór warunków reakcji i substratów, zarówno naturalnych jak i syntetycznych. Jedynie w tym artykule zastosowano międzynarodową jednostkę enzymatyczną do wyrażania wyników.

Referat wygłoszony przez M. Roth'a z Genewy poświęcony jest fluorymetrycznej metodzie oznaczania peptydaz. Autor podaje dokładną metodykę z użyciem syntetycznych substratów, które dają fluorescencję dopiero po rozszczepieniu. Mimo bardzo ciekawej i stosunkowo prostej metodyki trudno jednak w tej chwili mówić o przydatności tych badań dla celów klinicznych.

Szczególnie dokładnie omówiono izoenzymy dehydrogenazy mleczanowej (LDH). Omówiono zarówno naturę, metodykę, jak i znaczenie kliniczne oznaczania tego enzymu. Jednocześnie zwrócono

uwagę na trudności w interpretacji wyników, zalecając ostrożność w wyciąganiu wniosków; np. wykrywalność wczesnych stanów nowotworowych przez oznaczanie LDH jest ograniczona, chociaż z drugiej strony można tą drogą otrzymać wiele cennych informacji chociażby odnośnie skutków leczenia i ew. nawrotów. Autorzy podają także przypadki kliniczne z typowymi zmianami w zymogramach.

Jednym z obszerniejszych referatów był referat wygłoszony przez A. de Barbieri traktujący o enzymologii układu fibrynolitycznego. Autor opisuje schemat fibrynolizy i wpływ różnych czynników na przemianę plasminogenu w plasminę. Autor podaje również metodę izolowania urokinazy z moczu. Szkoda, że mimo zapowiedzi w tytule Autor nie podał znaczenia tych badań dla kliniki. Ograniczył się jedynie do ogólnikowych stwierdzeń, że badania te mogą mieć praktyczne znaczenie w opracowaniu patogenezy miażdżycy oraz w zabiegach na narządach miąższowych, gdzie rzeczywiście obserwuje się wzrost procesów fibrynolitycznych.

A. Dahlqvist bardzo wyczerpująco przedstawił stan badań nad jelitowymi dwusacharydazami zarówno u ludzi zdrowych, jak i u chorych z niedoborami tych enzymów. Autor przedstawia trudności w badaniu tych glikozydaz oraz poddaje krytyce dotychczas stosowane metody, szczególnie stosowanie sztucznych substratów. Stosowanie heteroglikozydów nie pozwala bowiem ocenić specyficzności enzymów. Poza tym stwierdzono, że istnieją w błonie śluzowej enzymy, które hydrolizują np. 6-bromo-3-metylo glikozyd, a nie hydrolizują dwucukrów odpowiadających mu konfiguracją. Syntetyczne substraty mogą być używane jedynie przy badaniu enzymów o dobrze poznanej specyficzności. Przedstawiono również fizjologię trawienia i wchłaniania dwucukrów, a także stany patologiczne wrodzone i nabyte. W artykule podano również sposoby oznaczania aktywności oraz sposoby rozdzielania jelitowych dwusacharydaz. Autor uwypukla zalety i wady poszczególnych metod, porównuje również nomenklaturę własną z nomenklaturą innych autorów.

Cztery referaty poświęcone są metodom histochemicznym z zastosowaniem mikroskopii elektronowej. Artykuły te ilustrowane są bardzo ładnymi zdjęciami.

Należy żałować, że nie zamieszczono referatów poświęconych zaburzeniom w metabolizmie aminokwasów, ograniczając się jedynie do bardzo zwięzłych streszczeń.

Oceniając książkę należy podkreślić jej przydatność dla biochemików klinicznych i lekarzy stosujących metody enzymologii w diagnostyce i w badaniach naukowych. Do referatów dołączony jest wyczerpujący spis literatury, co niewątpliwie podnosi wartość użytkową książki.

Witold Sendecki

CHEMICAL-BIOLOGICAL ACTIVITIES. March 22, 1965. Vol. 1. Number 6. Chemical Abstracts Service. A Publication of the American Chemical Society. Easton, Pa. U.S.A.

Amerykańskie Towarzystwo Chemiczne wydaje około 20 czasopism, w tym sześć czasopism podających aktualną dokumentację naukową. Są to: *Journal of Chemical and Engineering Data, Journal of Chemical Documentation, Chemical Abstracts, CA Section Groupings, Chemical Titles.* Czasopisma te wychodzą od kilku lat, poza tym od początku 1965 roku Towarzystwo zaczęło wydawać nowe czasopismo p.t. *Chemical-Biological Activities.* Jest to dwutygodnik o objętości około 120 stron dużego formatu informujący o treści bieżącej literatury naukowej dotyczącej aktywności biologicznej związków organicznych. Dokumentacją objęto około 300 czasopism, wśród których jedno polskie, Roczniki Chemii. Wydawcy położyli nacisk na szybkość informacji. W tym celu zastosowano maszyny elektronowe do sporządzania trzech indeksów: haseł, wzorów sumarycznych i autorów. O szybkości udostępniania informacji świadczyć może fakt, że w numerze *Chemical-Biological Activities* z 22 marca 1965, uwzględniono artykuły opublikowane w *Nature* z dnia 13 lutego 1965, czyli w 6 tygodni po ich opublikowaniu; nieliczne tylko streszczenia odnoszą się do artykułów opublikowanych pod koniec 1964 r. Czasopismo referuje wyłącznie te artykuły, które omawiają związki organiczne działające na systemy biologiczne z wyłączeniem systemów pochodzenia roślinnego. Informacje o każdym referowanym artykule są zgrupowane w schematyczny sposób

ułatwiający szybkie uzyskanie wiadomości o wynikach badań. Zawierają one dane o skutkach działania związków organicznych albo czynnika aktywnego na systemy biologiczne z uwzględnieniem gospodarza, tkanki itd.; każdemu związkowi organicznemu poświęcono oddzielny wiersz tekstu. Dla identyfikacji związków chemicznych o znanej budowie posłużono się ich numerami według specjalnego kodu Chemical Abstracts Service. Poza tym podano wzory strukturalne tych związków, które nie weszły do Merck Index (wydanie 1961) lub do wydawnictwa Amerykańskiego Towarzystwa Lekarskiego p.t. United States Adopted Names (US Pharmacopeal Convention Inc. New. York, 1964). Na specjalną uwagę zasługuje indeks haseł (Keyword-In-Context) sporządzony przez maszyny elektronowe. Wszystkie hasła, ułożone alfabetycznie, podano w wykazie łącznie z poprzedzającymi i następującymi po nich w tekście słowami, tj. w kontekście zdania w rozmiarze jednego pełnego wiersza, czyli podobnie jak to uczyniły inne wydawnictwa dokumentacyjne. Z samego więc już indeksu rzeczowego czytelnik może wnosić o rodzaju skatalogowanej informacji. Skorowidz haseł jest bardzo obszerny, ma objętość równą objętości wszystkich streszczeń. Według prospektów wydawnictwa na każdy referowany artykuł przypada średnio 25 - 30 haseł zawartych w trzech skorowidzach, co bardzo ułatwia i przyśpiesza znalezienie poszukiwanej informacji. Nazwy czasopism, z których pochodzą referowane artykuły, są podane w formie czteroliterowego szyfru (Coden for Periodical Titles, ASTM Special Technical Publication No 329, 1963 and Supplement I, No 329-S1 1964).

Należy podziwiać szybkość informacji osiągniętą w czasopiśmie. Zaleta ta jednak jest pomniejszona niezwykle wysoką ceną czasopisma, którego roczna prenumerata wynosi 750 dolarów; do sumy tej dochodzi opłata, która została uzależniona od liczby pracowników naukowych zatrudnionych przez prenumeratora, a wynosząca po 5 dolarów od osoby. Wydawca dokładnie precyzuje, co należy rozumieć pod nazwą pracownika naukowego. Przy 40 takich pracownikach koszt prenumeraty podnosi się do około tysiąca dolarów rocznie. Głównymi zaletami czasopisma są szybkość i jednolity, niemal schematyczny sposób informowania o aktywności biologicznej organicznych związków chemicznych.

Tadeusz Korzybski

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А. БЖЕЗИНЬСКИ и Б. ФИЛИПОВИЧ

СОДЕРЖАНИЕ ТИАМИНА И ЕГО ЭФИРОВ В СЕРДЕЧНОЙ МЫШЦЕ МОРСКОЙ СВИНКИ ПРИ ЭКСПЕРИМЕНТАЛЬНОЙ ДИФТЕРИЙНОЙ ТОКСЕМИИ

Резюме

В сердечной мышце здоровых морских свинок содержание общего тиамина составляет в среднем 6,6 μг/г мокрого веса ткани, из этого приблизительно 95% находится в виде кокарбоксилазы. При экспериментальной дифтерийной токсемии количество общего тиамина увеличивается на 25% в связи с увеличением уровня кокарбоксилазы. Это связано, повидимому, с увеличением нагрузки сердечной мышцы. Уменьшение содержания кокарбоксилазы, наблюдаемое в предсмертном периоде, можно считать одним из факторов приводящих к так наз. энергетическому пороку сердца.

А. БЖЕЗИНЬСКИ и Б. ФИЛИПОВИЧ

АКТИВНОСТЬ ТРАНСКЕТОЛАЗЫ ПРИ ЭКСПЕРИМЕНТАЛЬНОЙ ДИФТЕРИЙНОЙ ТОКСЕМИИ У МОРСКИХ СВИНОК

Резюме

У здоровых морских свинок наивысшая активность транскетолазы наблюдается в селезенке, а затем в легких, печени, почках, сердечной мышце и мозгу.

У морских свинок при интоксикации дифтерийным токсином наблюдается статистически достоверное увеличение активности транскетолазы сердечной мышцы (на 40%) и уменьшение ее активности в печени (на 40%), в мозгу (на 22%) и в селезенке (на 40%). В почках и в легких не наблюдались изменения активности транскетолазы.

Мария ЭРЕЦИНЬСКА и Людмила ШАРКОВСКА

ВЛИЯНИЕ ЭНЕРГИИ НА ВОССТАНОВЛЕНИЕ УБИХИНОНА В МИТОХОНДРИЯХ СЕРДЦА БЫКА

Резюме

Добавление сукцината к митохондриям сердца быка вызывает значительное восстановление митохондриального убихинона. АТР вызывает увеличение степени восстановления убихинона, тогда как факторы разобщающие фосфорилирование — наоборот, вызывают окисление убихинона. В митохондриях с частичным разобщением фосфорилирования и лишенных высокоэнергетических промежуточных продуктов путем длительной преинкубации

или под действием разобщающих факторов антимицин А не вызывает полного восстановления убихинона, которое может быть получено при добавлении АТР или KCN. Полученные результаты указывают на то, что восстановление убихинона зависит от фосфориляционного потенциала и что его положение в цепи реакций клеточного дыхания не является стойким.

Ю. БУЧЭК

ДЫХАНИЕ И ПОТРЕБЛЕНИЕ ВОДЫ В КЛУБНЯХ КАРТОФЕЛЯ ПЕРЕД И ПОСЛЕ ОКОНЧАНИЯ ПЕРИОДА ПОКОЯ

Резюме

1. Инкубация срезов картофеля после окончания периода покоя с α-кетоглютаратом, малатом, сукцинатом или фумаратом стимулирует потребление кислорода и воды; упомянутые соединения не оказывают влияния на дыхание и потребление воды в покоящихся клубнях картофеля.

2. Добавление к субстратам глютатиона или β-индолил-уксусной кислоты вызывает увеличение интенсивности дыхания покоящихся тканей и индуцирует одновременно потребление воды.

3. Обсуждается возможность влияния GSH и ауксина на метаболизм покоящихся клубней картофеля.

Галина АУГУСТЫНЯК и Я. АУГУСТЫНЯК

О СТРУКТУРЕ ОСНОВНОГО ПРОДУКТА ОБРАЗУЮЩЕГОСЯ ИЗ 2,5-ДИКЕТОПИПЕРАЗИНА В КИСЛОЙ СРЕДЕ

Резюме

Из 2,5-дикетопиперазина был получен основной продукт при помощи насыщения газообразным HCl его суспензии в диоксане, содержащем 2% воды. На основании проведенных исследований (восстановление соединения до пиперазина, гидролиз приводящий к образованию глицил-глицина и глицина, инфракрасный спектр, быстрое разложение в основной среде) авторы предполагают, что это соединение образуется в результате конденсации 2 или более промежуточных продуктов гидролиза 2,5-дикетопиперазина до глицил-глицина. Предполагается, что основные соединения, образующиеся из аминокислот при кислотном гидролизе, обладают сходной структурой.

М. ЖЫДОВО, В. МАКАРЕВИЧ, Е. УМЯСТОВСКИ и Ядвига ПУЖЫЦКА

ВЛИЯНИЕ ТЕМПЕРАТУРЫ НА ДЕЗАМИНАЦИЮ АМР КАТАЛИЗИРОВАННУЮ МЫШЕЧНЫМИ ЭКСТРАКТАМИ ТЕПЛОКРОВНЫХ И ХОЛОДНОКРОВНЫХ ЖИВОТНЫХ

Резюме

Исследовалось влияние температуры на скорость дезаминации АМР при концентрации субстрата равной 54 μм, при которой реакция протекает согласно кинетике первого порядка. В экстрактах из мышц трех видов теплокровных животных (крыса, кролик, голубь) наблю-

далось наличие острого оптимума температуры при 35°. В мышечных экстрактах холоднокровных животных (лягушка, форель, карп) не наблюдалось наличие такого оптимума, а скорость реакции при каждой высшей температуре не превышала при 10° двойной скорости.

V_{max} увеличивалось 7—16 раз при повышении температуры от 10° до 35° в обеих группах животных.

С. КСЕНЖНЫ, В. АРДЭЛЬТ, А. З. БУДЗЫНЬСКИ,
Изабелла НЕДЗВЕДЗКА-НАМЫСЛОВСКА и Эльжбета ВОЙТЭЦКА-ЛУКАСИК

НЕКОТОРЫЕ СВОЙСТВА ПРОДУКТОВ ДЕГРАДАЦИИ ЭЛАСТИНА

Резюме

α- и β-эластин был получен при помощи обработки щавелево-уксусной кислотой нерастворимого эластина, полученного из шейной связки быка. Оба белки осаждались танином в широком диапазоне pH; наилучшие результаты осаждения получаются при pH $\leqslant 1$ и между pH 3,6 и 5,5. Хлорид цетилпиридина предотвращает осаждение α-эластина в концентрации в десять раз меньше чем концентрация предотвращающая осаждение β-эластина.

Оба эти белки окрашиваются нигрозином при pH ниже 3,1, при высшем pH окрашивается только α-эластином. Различная электрофоретическая подвижность этих белков в крахмальном желе при pH 3,0 позволяет разделить эти белки.

Только в случае α-эластина граничные числа вязкости зависят от pH, с минимумом при pH 4,5.

Целина ЯНИОН и Д. ШУГАР

МУТАГЕННАЯ АКТИВНОСТЬ ГИДРОКСИЛАМИНА: РЕАКЦИЯ С АНАЛОГАМИ ЦИТОЗИНА, 5(6)-ЗАМЕЩЕННЫМИ СОЕДИНЕНИЯМИ ЦИТОЗИНА И НЕКОТОРЫМИ 2-КЕТО-4-ЭТОКСИПИРИМИДИНАМИ

Резюме

Исследовалось влияние гидроксиламина в водной нейтральной среде на цитозин, 5-замещенные соединения цитозина, их гликозиды, соединения цитозина алкилированные в аминогруппе и на модельное соединение 2-кето-4-этоксипиримидин.

Реакция гидроксиламина с цитозином состоит в присоединении этого соединения к связи 5,6, причем аминогруппа тотчас же замещается второй молекулой гидроксиламина. В кислой среде молекула гидроксиламина из связи 5,6 отделяется.

У соединений группы цитозина с замещением в положении C_5 и у их гликозидов присоединение гидроксиламина к связи 5,6 не происходит, получается только замена аминогруппы на гидроксиламиногруппу. Эта реакция полностью количественная. Соединения цитозина замещенные в позиции 6 ведут себя аналогично.

Реакции исследовались при помощи ряда реагирующих с гидроксиламином аналогов 4-этокси-2-летопиримидинов.

В связи с мутагенной активностью гидроксиламина обсуждаются свойства продуктов реакции цитозина и 5-метилцитозина, а также предлагаются схемы опытов для проверки мутагенной роли реакции присоединения и замещения.

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КОЛОРИМЕТРИЧЕСКОЕ ОПРЕДЕЛЕНИЕ АМИНОПЕПТИДАЗ ПРИ ПОМОЩИ НИТРИЛОВ ДИПЕПТИДОВ

Резюме

1. Получены шесть нитрилов дипептидов и их фталоилопроизводных, а также *N*-(карбоксибензоил)-лейцилаланилнитрил.

2. Нитрилы дипептидов можно применять в качестве субстратов для колориметрического определения аминопептидазной активности в сыворотке, в моче и в тканевых экстрактах.

3. Исследовалось влияние ионов металлов, пептидов и pH на аминопептидазную активность человеческой сыворотки, экстракта почки свиньи и очищенной лейцил-аминопептидазы.

4. Определялась аминопептидазная активность сыворотки больных и здоровых людей.

5. Результаты определения ферментативной активности по отношению к различным дипептидам, нитрилам и лейцил-β-нафтиламиду позволяют считать, что соединения обоих типов пригодны для дифференцировки аминопептидаз.

Зофия ЛЯСОТА

ПОТРЕБЛЕНИЕ КИСЛОРОДА И ВЫДЕЛЕНИЕ АММИАКА НОРМАЛЬНЫМИ И ОБЛУЧЕННЫМИ ЛУЧАМИ ЯЙЦАМИ BOMBYX MORI

Резюме

1. В нормальных яйцах дыхание в постдиапаузальном периоде зависит от скорости развития. Ускорение дыхания вызываемое повышением температуры касается, главным образом, более позднего периода развития, после бластокинеза.

2. Дозы излучения полностью тормозящие вылупливание гусениц не вызывали мгновенной смерти яиц. Облучение дозой 200 kr блокировало дыхание на уровне соответствующем стадии бластули, а облучение дозой 20 kr — на уровне соответствующем поздней бластули.

3. Доза 20 kr замедляла увеличение интенсивности дыхания в постдиапаузальном периоде. Температура и длительность перерыва между облучением и инкубацией влияли на это замедление, но не изменяли конечного эффекта.

4. Не было обнаружено выделение аммиака ни в нормальных, ни в облученных яйцах.

М. ГИЛЛЯР

РАЗОБЩЕНИЕ ОКИСЛИТЕЛЬНОГО ФОСФОРИЛИРОВАНИЯ ОСНОВНЫМИ БЕЛКАМИ

Резюме

Исследовалось разобщающее влияние протамина, основного митохондриального белка и рыбонуклеазы на окислительное фосфорилирование в митохондриях печени крысы.

Алиция ДРАБИКОВСКА и Людмила ШАРКОВСКА

ВОССТАНОВЛЕНИЕ УБИХИНОНА В МИТОХОНДРИЯХ ПЕЧЕНИ, СВЯЗАННОЕ С ОКИСЛЕНИЕМ ХОЛИНА

Резюме

1. Холин добавленный к суспензии митохондриев вызывает восстановление убихинона. Возможность участия альдегида бетаина в восстановлении убихинона холином была исключена путем применения семикарбазида.

2. В митохондриях лишенных NAD убихинон восстанавливается как холином, так и альдегидом бетаина. Окисление альдегида бетаина катализируется, повидимому, специфическим ферментом не требующим участия NAD.

3. Холин и альдегид бетаина восстанавливают экзогенный убихинон в присутствии митохондрий или в присутствии препарата растворимой дегидрогеназы холина.

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СИНТЕЗ И ДЕГРАДАЦИЯ АЛКАЛОИДОВ В ОНТОГЕНЕЗЕ ЛЮПИНА LUPINUS ANGUSTIFOLIUS

Резюме

1. Исследовался алкалоидный состав Lupinus angustifolius в разных периодах развития этого растения.

2. На основании полученных результатов авторы предложили схему биосинтеза алкалоидов в Lupinus angustifolius.